

RICE UNIVERSITY

**Escort protein regulation of the human mitochondrial molecular
chaperone mtHsp70**

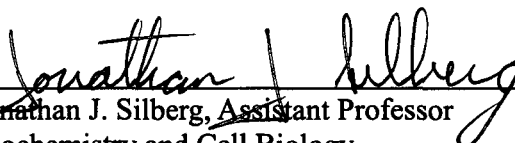
by


Peng Zhai


A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

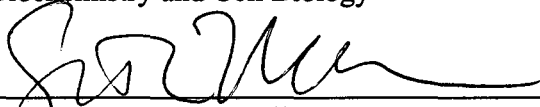
Doctor of Philosophy

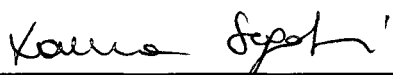
APPROVED, THESIS COMMITTEE:


Jonathan J. Silberg, Assistant Professor
Biochemistry and Cell Biology


Yizhi Jane Tao, Assistant Professor
Biochemistry and Cell Biology


Yousif Shamoo, Associate Professor
Biochemistry and Cell Biology


Seiji Matsuda, E. Dell Butcher Professor
Chemistry; Professor, Biochemistry and Cell
Biology


Laura Segatori, T.N. Law Assistant Professor
Chemical and Biomolecular Engineering;
Assistant Professor, Bioengineering

HOUSTON, TEXAS
MAY 2010

UMI Number: 3421174

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

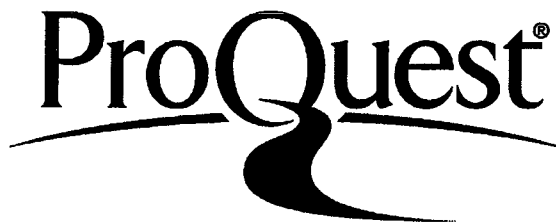
In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI 3421174

Copyright 2010 by ProQuest LLC.

All rights reserved. This edition of the work is protected against unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Escort protein regulation of the human mitochondrial molecular chaperone mtHsp70

by

Peng Zhai

Mitochondrial Hsp70 molecular chaperones (mtHsp70) are nuclear-encoded proteins that are needed for the import of proteins into the mitochondrial matrix, biogenesis of Fe/S-clusters involved in energy metabolism, and stress response folding. Unlike the Hsp70 homologs found in other subcellular compartments, two of the mtHsp70 homologs in yeast require a specialized Hsp70 escort protein (Hep1) to maintain their solubility and function. To better understand the role that escort proteins play in regulating their cognate chaperones, I have characterized the regulation of human mtHsp70 by the human Hep, an ortholog of Hep1. In my thesis research, I show that human Hep is localized to the mitochondria of tissue culture cells, like mtHsp70. In addition, I demonstrate that human Hep enhances the solubility of mtHsp70 upon overexpression in *Escherichia coli* through an interaction with its N-terminal ATPase domain (70^{ATPase}). Chromatography, fluorescence, and copurification analysis using recombinant proteins indicate that Hep binds most tightly to nucleotide-free mtHsp70 and 70^{ATPase} . While this complex is destabilized by the presence of ADP and ATP, ATPase measurements indicate that Hep binds to ATP-bound mtHsp70 (and 70^{ATPase}) and stimulates the steady-state rate of ATP hydrolysis, implicating a role for Hep in directly regulating the mtHsp70 chaperone

reaction cycle. To identify Hep residues that are critical for chaperone regulation, I also carried out an alanine mutagenesis scan of charged residues in a tryptophan-free mutant (W115I) of human Hep and assessed the effect of each mutation on Hep and mtHsp70 interactions. *In vitro* binding studies identified three mutations (R81A, H107A, and D111A) with decreased affinity to nucleotide-free chaperone, and ATPase measurements revealed that one of these mutants (H107A) fails to elicit an increase in the steady-state activity of 70^{ATPase}. Yeast complementation studies further revealed that Hep supports the growth of $\Delta hep1$ *Saccharomyces cerevisiae* like yeast Hep1, whereas a Hep-H107A cannot complement $\Delta hep1$ yeast. These findings demonstrate that human Hep is a functional ortholog of yeast Hep1, and provide the first evidence that escort proteins directly regulate the catalytic activity of nucleotide-bound chaperones. Furthermore, they identify a histidine conserved in all mitochondrial and plastid escort proteins as critical for the regulation of human mtHsp70.

Acknowledgements

The work presented here would not have been possible without the guidance of Dr. Jonathan Joff Silberg. Joff is a great mentor. His enthusiasm and demand for excellence deeply impress me. He always comes up with great new ideas on the project, which has been a constant source of encouragement for me especially in the time of difficulties. He also cares a lot about my career and provides numerous valuable advices. With his generous reference, I successfully received a number of awards and travel fellowships to national and international conferences. More importantly, it wouldn't have been possible for me to be admitted to my post-doctoral training position at Nation Institute of Health without his support. I am also grateful to my committee: Dr. Tao, Dr. Shamoo, Dr. Matsuda, and Dr. Segatori. They gave me great advice during progress reviews and were always supportive.

I would like to thank my current and previous lab members: Shirley, Peter, Crystal, Rochael, Kevin, Michael, and Rui. Shirley provides countless technical support. She also prepares a lot of materials, which makes my work much easier. Peter and I became good friends since the graduate school interview. We always have great discussions on our projects, which lead to a number of wonderful ideas. Crystal started the escort protein project, which provides a great platform for me to continue. I am so grateful that everyone in the lab is so nice and easy to work with. The pleasant working environment is definitely a big plus for my graduate school study.

I would like to thank Dr. Gustin and Dr. McNew for their directions on yeast genomics studies. My yeast complementation assay wouldn't have been possible without their

support as I knew nothing about yeast before. I would also like to thank Dr. Raphael and Dr. McGuire for their generous help on mammalian cell culture and imaging. I am also grateful to my friends Todd, Tom, Shuya, Michael, and Peter for their help to proofread my thesis.

I would like to thank my family, especially my wife, Yao. Her loving support and encouragement have helped me overcome many of challenges. She also provides me a beautiful and warm home, which always makes me feel so relax after extensive work. My parents love me so much and their high expectation on me has always been a source of power to push me forward. I will do my best to keep them proud.

Last but not least, I would like to thank everyone in the department. I could not count how many times I went to other labs for assistance and is welcomed with generosity. The friendly environment in the department makes the hard graduate school study much easier.

Table of Contents

Chapter 1. Introduction.....	1
1.1. Molecular chaperones and protein folding.....	1
1.2 The 70-kDa heat shock protein family (Hsp70s).....	2
1.2.1. Initial discovery of hsp70 molecular chaperones.....	2
1.2.2. Domain structure of Hsp70 chaperones.....	4
1.2.3. Evolution of hsp70 chaperones.....	9
1.2.4. The prototypical DnaK, DnaJ, and GrpE system in <i>E. coli</i>	10
1.2.5. Hsp70 proteins in yeast and metazoans.....	12
1.3. Functions of yeast mitochondrial Hsp70 chaperones.....	13
1.3.1. Yeast Ssc1 exhibits classical chaperone activity within mitochondria.....	15
1.3.2. Yeast Ssc1 is essential for the protein import into mitochondria matrix.....	15
1.3.3. Ssq1 is needed for mitochondrial iron-sulfur cluster biogenesis.....	17
1.4. Functions of metazoan mitochondrial Hsp70 chaperones.....	18
1.4.1. mtHsp70 is subject to post-translational modifications.....	19
1.4.2. Differences in human and yeast mitochondrial cochaperones.....	19
1.4.3. Human mtHsp70 has cytosolic functions.....	21
1.5. Human mtHsp70 and human diseases.....	21
1.5.1. A mutation in zebrafish mtHsp70 causes a blood development defect.....	21
1.5.2. Changes in mtHsp70 expression are correlated with multiple diseases.....	22
1.6. Mitochondrial Hsp70 Escort Proteins.....	24
1.6.1. Discovery of the Hsp70 Escort Protein Hep1.....	24

1.6.2. Hep1 depletion causes chaperone-like defects.....	25
1.6.3. Evolutionary conservation of escort proteins.....	27
1.6.4. Structural studies of <i>S. cerevisiae</i> Hep1.....	29
1.7. Summary and overview of my thesis research.....	31

Chapter 2. The human escort protein Hep binds to the ATPase domain of mitochondrial Hsp70 and regulates ATP hydrolysis.....	33
2.1. Introduction.....	34
2.2. Experimental procedures.....	36
2.2.1 Materials.....	36
2.2.2. Vectors.....	36
2.2.3 Solubility analysis.....	38
2.2.4. Protein expression and purification.....	39
2.2.5. Gel filtration chromatography.....	40
2.2.6. Cell culture.....	41
2.2.7 ATPase measurements.....	41
2.2.8. Analytical methods.....	42
2.3. Results.....	42
2.3.1. Domain requirements for mtHsp70 insolubility.....	42
2.3.2. Hep-EGFP is localized to mitochondria.....	43
2.3.3. Hep enhances mtHsp70 solubility.....	47
2.3.4. Protein expression and purification.....	49
2.3.5. Gel filtration analysis.....	53
2.3.6. Nucleotide effects on Hep binding.....	57

2.3.7. ATPase activity	58
2.4. Discussion.....	65

Chapter 3. A conserved histidine in human Hep is required for

stimulation of mitochondrial Hsp70 ATPase activity.....	69
3.1. Introduction.....	70
3.2. Experimental procedures.....	72
3.2.1. Materials.....	72
3.2.2. Vectors.....	72
3.2.3. Protein Purification.....	74
3.2.4. Escort activity screen.....	75
3.2.5. Fluorescence analysis.....	76.
3.2.6. Copurification of protein complexes.....	76.
3.2.7. ATPase assays.....	77
3.2.8. Surface plasmon resonance.....	77
3.2.9. Zinc assay.....	77
3.2.10. Yeast complementation.....	78
3.2.11. Analytical methods.....	79
3.3. Results.....	79
3.3.1. A tryptophan-free Hep mutant.....	79
3.3.2. Hep mutant effects on 70 ^{ATPase} fluorescence.....	86
3.3.3. Hep mutant effects on 70 ^{ATPase} activity.....	90
3.3.4. Escort activity of Hep mutants.....	91

3.3.5. SPR analysis of binding.....	93
3.3.6. Zinc content of Hep.....	95
3.3.7. Human Hep complements yeast Δ hep1.....	95
3.3.8. Escort activity of human Hep truncation mutants.....	98
3.3.9. Zn^{2+} is required for the solubility of yeast Hep1.....	98
3.3.10. Zn^{2+} is not required for the solubility of human Hep.....	101
3.4. Discussion.....	104

Chapter 4. Conclusions

4.1. Escort proteins stimulate Hsp70 ATPase activity.....	108
4.2. Human Hep and yeast Hep1 are functional orthologs.....	112
4.3. Mechanism of human Hep and mtHsp70 binding.....	114
4.4 Zinc regulation of escort proteins.....	117
4.5 Human Hep regulation of mtHsp70 as a therapeutic target.....	118

Bibliography.....	120
--------------------------	------------

List of Figures and Tables

Figure 1.1. ATP binding and hydrolysis regulate chaperone and substrate binding.....	6
Figure 1.2. Structures of the Hsp70 ATPase and peptide binding domains.....	8
Figure 1.3. Cochaperone regulation of DnaK.....	11
Figure 1.4. Functions of mtHsp70 chaperones.....	14
Figure 1.5. Sequence alignment of escort proteins.....	28
Figure 1.6. NMR structure of the trypsin-resistant core domain of Hep1.....	30
Figure 2.1. Chimeras and truncation constructed.....	44
Figure 2.2. Solubility of mtHsp70, mtHsp70-HscA chimeras, and 70ATPase.....	45
Figure 2.3. Mitochondrial localization of human Hep.....	46
Figure 2.4. SDS-PAGE analysis of human Hep escort activity.....	48
Figure 2.5. SDS-PAGE analysis of yeast Hep1 and human Isu2 escort activity.....	50
Figure 2.6. Nucleotide effects on mtHsp70 oligomerization.....	52
Figure 2.7. Hep inhibits mtHsp70 oligomerization.....	54
Figure 2.8. Hep binds to the mtHsp70 ATPase domain.....	56
Figure 2.9. Hep stimulates the ATPase activity of 70ATPase.....	59
Figure 2.10. Effect of Hep concentration on the ATPase activity of 70ATPase.....	60
Figure 2.11. Calorimetric analysis of Hep and mtHsp70 binding.....	61
Figure 2.12. Hep stimulates full-length mtHsp70 ATPase activity.....	63
Figure 2.13. Effect of Hep concentration on the ATPase activity of mtHsp70.....	64
Figure 3.1. Alignment of the sequences of yeast Hep1 and human Hep showing conserved charged residues.....	80

Figure 3.2. Hep-W115I decreases 70ATPase tryptophan fluorescence.....	82
Figure 3.3. Effect of increasing concentrations of Hep-W115I on the fluorescence of 70ATPase.....	83
Figure 3.4. Effect of W115I mutation on escort activity.....	84
Figure 3.5. Effect of W115I and H107A mutations on Hep stimulation of 70ATPase activity.....	85
Figure 3.6. Yeast Hep1 stimulation of human mtHsp70 ATPase activity.....	87
Table 3.1. Stimulation of 70ATPase activity by escort proteins.....	88
Figure 3.7. Effects of mutations on Hep binding to nucleotide-free 70ATPase.....	89
Figure 3.8. SDS-PAGE analysis of Hep mutant escort activity.....	92
Figure 3.9. SPR analysis of chaperone and escort protein binding.....	94
Figure 3.10. Far-UV circular dichroism spectra of Hep variants.....	96
Figure 3.11. Hep complementation of yeast $\Delta hep1$	97
Figure 3.12. Escort activity of human Hep truncation mutants.....	99
Figure 3.13. Effect of zinc on <i>S. cerevisiae</i> Hep1 solubility.....	100
Figure 3.14. Zn ²⁺ displacement leads to aggregation of <i>S. cerevisiae</i> Hep1.....	102
Figure 3.15. Effect of zinc on human Hep solubility.....	103
Figure 4.1. Possible mechanism for the initial fast phase of the biphasic kinetics in the ATPase assay of mtHsp70 and Hep.....	110
Figure 4.2. Possible mechanism for the slow phase of the biphasic kinetics in the ATPase assay of mtHsp70 and Hep.....	111
Figure 4.3. NMR structure of the region of <i>S. cerevisiae</i> Hep1 corresponding to the defined smallest function subdomain of human Hep.....	116

List of Abbreviations

5'-FOA, 5'-fluoroorotic acid

70^{ATPase}, mtHsp70 ATPase domain

70-A, mtHsp70-HscA chimera having mtHsp70 ATPase domain

A-70, HscA-mtHsp70 chimera having HscA ATPase domain

ADP, Adenosine-5'-diphosphate

ATP, Adenosine-5'-triphosphate

ATP γ S, Adenosine-5'-gamma-thiotriphosphate

CD, Circular dichroism

CDR, critical deleted region

C. elegans, *Caenorhabditis elegans*

DTT, dithiothreitol

E. coli, *Escherichia coli*

Fe-S-cluster, Iron-sulfur-cluster

G418, gentamicin sulfate

HCC, hepatocellular carcinoma

Hep, Hsp70 Escort Protein

Hsc, heat-shock cognate protein

HSE, heat-shock element

Hsp, heat-shock protein

HSQC, heteronuclear single quantum coherence

IPTG, isopropylthio- β -D-galactoside

JDP, J-domain protein

LB, luria broth

MALDI, Matrix-assisted laser desorption/ionization

MDS, myelodysplastic syndrome

MMTS, methyl methanethiolsulphonate

MtHsp70, mitochondria Hsp70

MTS, mitochondria targeting sequence

NBD, nucleotide-binding domain

NEF, nucleotide exchange factor

NMR, nuclear magnetic resonance

PAC, P1-derived artificial chromosome

PAR, 4-(2-pyridylazo) resorcinol

PBD, peptide-binding domain

PD, Parkinson's disease

PMSF, phenylmethanesulphonyl fluoride

ROS, Reactive oxygen species

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

S. cerevisiae, *Saccharomyces cerevisiae*

SPR, surface Plasmon resonance

Chapter 1

Introduction

1.1. Molecular chaperones and protein folding.

Over fifty years ago, Christian Anfinsen demonstrated that the tertiary structures of proteins are encoded by their primary amino acid sequence (Anfinsen et al., 1955). He showed that chemically-denatured proteins can refold spontaneously into a functional conformation upon dilution into a solution lacking denaturant (Anfinsen, 1973; Dobson and Karplus, 1999). Unfortunately, protein folding in the cellular environment can be far more complicated than that of the proteins that Anfinsen studied. Highly concentrated macromolecules (such as proteins, nucleic acids, and lipids) are present within cells that can interact with proteins during translation. These biomolecules can interfere with proteins folding to their native state and lead to undesirable off-pathway reactions, such as protein misfolding and aggregation (Ellis, 2001), which can at times be toxic to cells (Chaudhuri and Paul, 2006; Trzesniewska et al., 2004). Protein folding is further complicated because it cannot occur in a concerted manner during translation, as in Anfinsen's experiments (Anfinsen et al., 1955). As the ribosome synthesizes a protein, the N-terminal residues are unstructured as they go through the exit channel of the ribosome (Mankin, 2006; Nissen et al., 2000), and they expose aggregation prone hydrophobic residues before they can be buried into the hydrophobic core of the protein with residues that have not been translated yet (Deuerling and Bukau, 2004; Frydman, 2001).

To deal with this issue, molecular chaperones have evolved to prevent the aggregation and misfolding of newly synthesized and stress-denatured proteins (for reviews, *see* Deuerling and Bukau, 2004; Frydman, 2001; Hendrick and Hartl, 1995; Young et al., 2004). In most cases, chaperones are thought to inhibit these off-pathway reactions by decreasing the effective concentrations of unfolded proteins in the cell, thereby giving proteins a chance to fold under conditions that are more favorable for achieving the native state (Hendrick and Hartl, 1995; Young et al., 2004). While most chaperones are thought to prevent off-pathway reactions, there are examples of chaperones that function as foldases (Tang et al., 2006). A diverse array of chaperones have been discovered in prokaryotes and eukaryotes. They are typically classified into families based on their size, with the most common families having molecular weights of 60 kDa (chaperonins), 70 kDa (Hsp70), and 90 kDa (Hsp90) (Gottesman and Hendrickson, 2000; Hendrick and Hartl, 1995; Young et al., 2004). Molecular chaperones are also found in most cellular compartments of eukaryotic cells and have evolved specialized functions in addition to chaperone activity (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002; Neupert and Brunner, 2002; Young et al., 2003).

1.2 The 70-kDa heat shock protein family (Hsp70s).

1.2.1. Initial discovery of hsp70 molecular chaperones.

Concomitant with Anfinsen's studies, Ritossa observed that non-lethal heat treatments of cells led to chromosome puffs in fruit flies (Ritossa, 1962), and subsequent studies revealed that these puffs were due to the upregulation of select proteins defined as a "heat shock proteins" (Moran et al., 1978; Tissieres et al., 1974). Further studies showed that a

mild heat treatment could increase the resistance of these cells to subsequent heat shock, which would otherwise have been lethal (Gerner et al., 1976; Henle et al., 1978; Jaattela et al., 1989; Petersen and Mitchell, 1981; Sapareto et al., 1978). This “vaccine”-like effect was attributed to an increased expression of heat shock proteins having a molecular weight of 70 kDa (Hsp70), which have since been shown to exhibit chaperone activity that aids protein folding (Johnston and Kucey, 1988; Li et al., 1991; Li and Werb, 1982; Pelham, 1984; Riabowol et al., 1988). Homologs of the original proteins discovered are now classified as Hsp70 family members, although some are constitutively expressed and do not exhibit changes in expression upon cellular stress. These family members are often called heat shock cognate 70 (Hsc70) proteins (Lelivelt and Kawula, 1995). Biochemical and genetic studies of Hsp70 (and Hsc70) proteins have provided evidence that these proteins can aid in cellular processes other than protein folding. Hsp70 family members have been shown to participate in the degradation of proteins (Hohfeld et al., 2001), the translocation of proteins into subcellular compartments such as the endoplasmic reticulum and mitochondria (Neupert and Brunner, 2002; Ryan and Pfanner, 2001), clathrin uncoating of vesicles (Ryan and Pfanner, 2001), and iron-sulfur cluster biosynthesis reactions (Vickery and Cupp-Vickery, 2007). Hsp70 molecular chaperones are also thought to have roles in signal transduction (Gaestel, 2006), the cell cycle (Helmbrecht et al., 2000), differentiation, and apoptosis (Garrido et al., 2003).

In *Escherichia. coli*, the prototypical Hsp70 molecular chaperone DnaK has been estimated to assist in the *de novo* folding of 5-18% of newly synthesized proteins (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002). Because eukaryotes have proteins with an increased average size compared with prokaryotes (Cagney et al., 2003), this percentage

is predicted to be even higher in multicellular organisms (Mayer and Bukau, 2005). In addition, DnaK is thought to interact with an even larger percentage of the *E. coli* proteome subsequent to heat shocks (Mayer and Bukau, 2005). Biochemical studies with *E. coli* DnaK have provided evidence that this occurs because Hsp70 chaperones prevent protein aggregation by transiently binding to hydrophobic polypeptide motifs (Genevaux et al., 2007), which occur on average approximately every 30 to 40 residues within soluble bacterial protein (Rudiger et al., 1997). Binding to these hydrophobic patches is thought to compete with the association of unfolded proteins in the cell that can lead to off-pathway aggregation (Mayer and Bukau, 2005). Hsp70 family members are thought to both passively and actively assist in protein folding. On one hand, the association of Hsp70 chaperones with unfolded proteins lowers the concentration of aggregation-prone polypeptides in the cell and allows them to fold into their native state under more favorable conditions (Ben-Zvi and Goloubinoff, 2001). On the other hand, formation of Hsp70-substrate complexes have been proposed to induce “local unfolding” and help proteins overcome kinetic energy barriers along the folding path (Ben-Zvi and Goloubinoff, 2001).

1.2.2. Domain structure of Hsp70 chaperones.

All Hsp70 chaperones contain an N-terminal ATPase domain (also designated as nucleotide-binding domain (NBD)) that hydrolyzes ATP and a C-terminal domain that binds polypeptide substrates, designated the peptide-binding domain (PBD). Biochemical studies have shown that the affinity of the PBD for substrates is regulated by nucleotide-induced conformational changes in the ATPase domain (Mayer and Bukau, 2005;

Morano, 2007). As illustrated in Figure 1.1, ATP-binding leads to a tense (T) conformational state with a lower affinity for protein substrates, and ATP hydrolysis leads to the formation of a relaxed (R) state which binds substrates more tightly and exhibits slower substrate dissociation and association rates (Genevaux et al., 2007; Mayer and Bukau, 2005). The interconversion of Hsp70 family members between their R and T states are subject to regulation by several families of auxiliary cochaperones, which are required for these molecular chaperones to fulfill their diverse cellular functions.

Auxiliary cochaperones have been discovered that stimulate the rate of ATP hydrolysis and formation of the R state, like J-type cochaperones (DnaJ and Hsp40) (Karzai and McMacken, 1996; Laufen et al., 1999; Liberek et al., 1991). In addition, cochaperones have been characterized that stimulate ADP release and formation of the T state, like GrpE and Bag-1 nucleotide exchange factors (Brehmer et al., 2001; Harrison et al., 1997).

Structures are available for the individual ATPase (Flaherty et al., 1990; Zhang and Zuiderweg, 2004) and peptide-binding (Cupp-Vickery et al., 2004; Mayer et al., 2000; Zhu et al., 1996) domains of multiple Hsp70 family members, as well as fragments containing portions of both domains (Revington et al., 2005; Swain et al., 2007). Figure 1.2A shows that the atomic structure of the ATPase domain of ADP-bound bovine hsc70 (Flaherty et al., 1990). This domain consists of two lobes separated by a deep cleft, each of which can be further divided into the subdomains IA, IB, IIA, and IIB (Flaherty et al., 1990). Nucleotides as well as one Mg^{2+} and two K^{+} ions sit on the bottom of the cleft, with all four subdomains making contact with the bound nucleotide (Flaherty et al., 1990). Nuclear magnetic resonance (NMR) studies of bovine Hsc70 have revealed that in solution the orientations of lobes I and II differ by up to 10° compared to that observed in

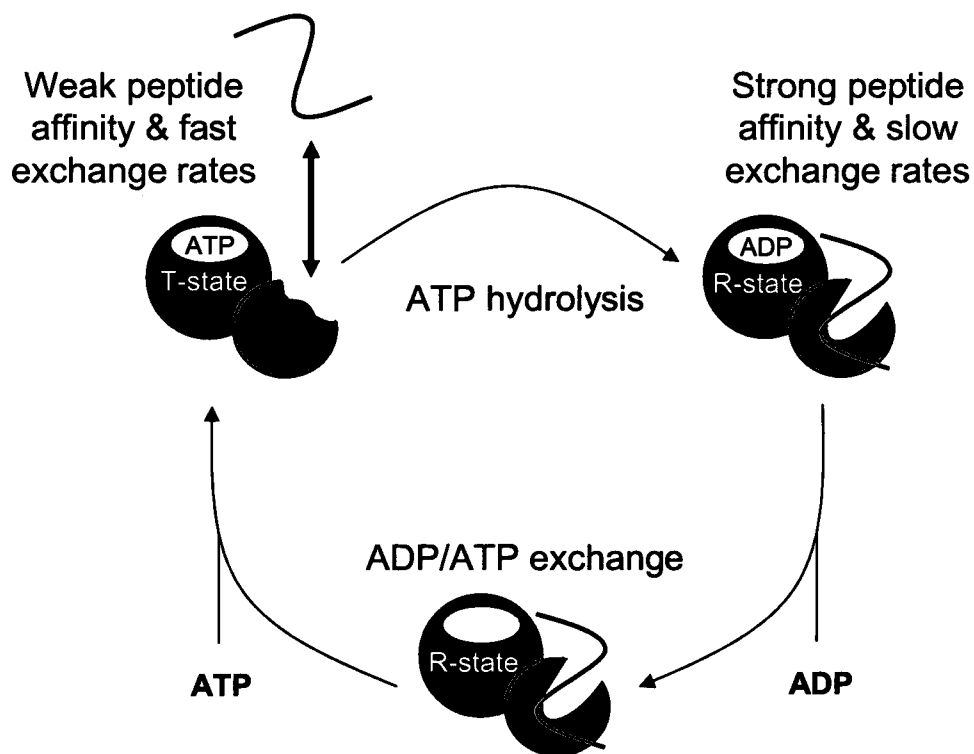


Figure 1.1. ATP binding and hydrolysis regulate chaperone and substrate binding. The association and dissociation of Hsp70 chaperones and their polypeptide substrates is regulated by the nucleotide present in their ATPase domain. The ATP-bound (T-state) exhibits low affinity for peptide substrates and fast exchange rate, whereas the ADP-bound (R state) exhibits high affinity and slow substrate exchange rates.

the crystal structure (Zhang and Zuiderweg, 2004). In solution, the residues linking the two lobes and subdomains are not completely assigned, suggesting that these regions are also flexible. These observations have also led to the hypothesis that cochaperone regulation of chaperone and nucleotide interactions (*i.e.*, ATP hydrolysis and nucleotide exchange) may arise because cochaperone binding to the ATPase domain causes a similar conformational change (Zhang and Zuiderweg, 2004).

Structural studies have revealed that the C-terminal PBD consists of a pair of four-stranded β -sheets (Figure 1.2B) that form a β -sandwich and a pair of interacting α -helices that form a lid that covers the pocket (Zhu et al., 1996). Peptide-bound structures indicate that polypeptide substrates reside within the pocket of the β -sandwich in an extended conformation (Cupp-Vickery et al., 2004; Zhu et al., 1996). Currently, the exact role of the C-terminal helical lid is not known. ATP-binding to the ATPase domain leads to a movement of the α -helical lid away from the substrate binding pocket, suggesting that this conformational change helps regulate substrate-binding kinetics (Zhu et al., 1996). However, biochemical and structural studies have provided evidence that substrate binding is mainly dictated by changes in the conformation of the hydrophobic pocket in the β -sandwich that directly contacts peptide substrates (Mayer et al., 2000; Pellicchia et al., 2000). For example, a mutation within the β -sandwich pocket in full-length DnaK causes a >20-fold reduction of affinity for substrates, and this mutant cannot complement the growth defect of a $\Delta dnaK$ *E. coli* at 40°C (Mayer et al., 2000). In contrast, a DnaK truncation mutant containing an ATPase domain fused to the β -sandwich (but lacking the lid of the PBD) exhibits a minor decrease (5 fold) in affinity for peptide substrates (–ATP) compared with full-length DnaK (Pellicchia et al., 2000), and this truncation mutant

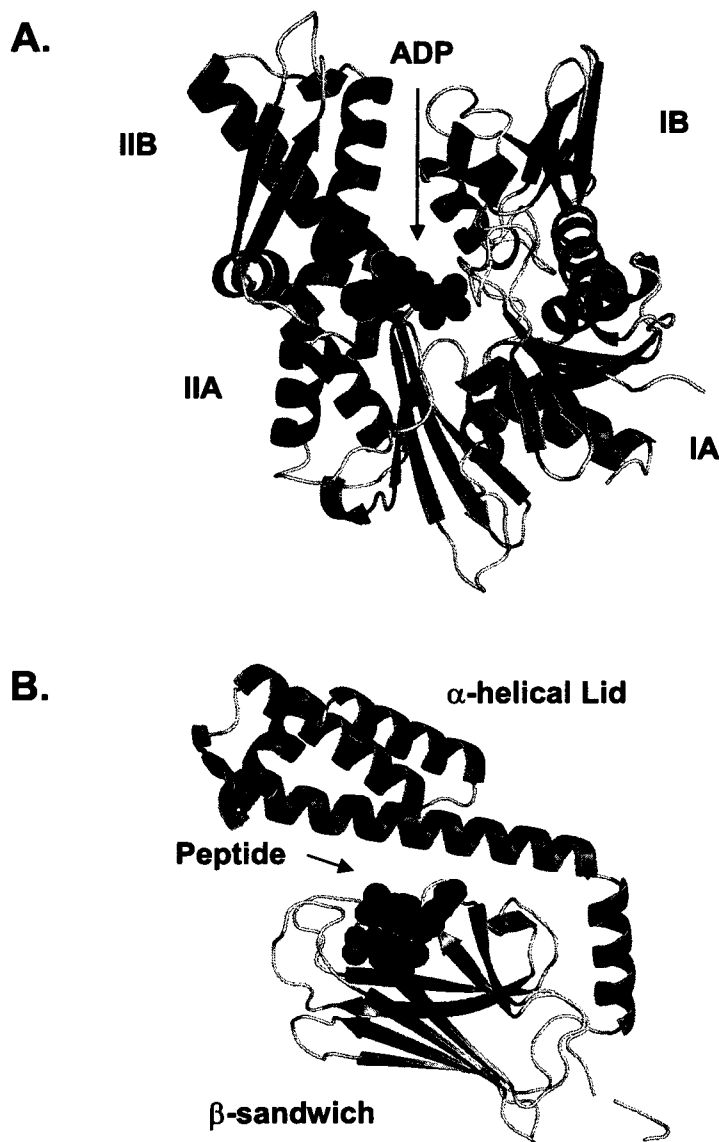


Figure 1.2. Structures of the Hsp70 ATPase and peptide binding domains.

A. Ribbon representation of the crystal structure of the *bovine* Hsp70 ATPase domain in complex with ADP (PDB = 3HSC). The four subdomains (IA, IB, IIA, and IIB), nucleotide-binding cleft, and bound ADP (red) are labeled. *B.* Ribbon representation of the crystal structure of *E. coli* DnaK (PDB = 1DKX) PBD in a complex with a peptide. The α -helical lid, the β -sandwich substrate binding pocket, and bound peptide substrate (red) are labeled.

exhibits decreased affinity for substrates in the presence of ATP, like full-length DnaK (Pellecchia et al., 2000) In addition, this mutant complements the growth defect of $\Delta dnaK$ *E. coli* under heat shock conditions (40°C) (Mayer et al., 2000).

1.2.3. Evolution of hsp70 chaperones.

Hsp70 family members are found in most prokaryotes, with *E. coli* having three family members, including DnaK, HscA (Hsc66), and HscC (Genevaux et al., 2007). Hsp70 homologs also have been found in the cytosol, mitochondria, endoplasmic reticulum, and lysosomes of eukaryotes, with some subcellular compartments have as many as half a dozen hsp70 homologs (Daugaard et al., 2007; Mayer and Bukau, 2005; Werner-Washburne and Craig, 1989). A comparison of hsp70 primary amino acid sequences indicates that their N-terminal ATPase domains exhibit a higher level of sequence conservation than their C-terminal domains (Brocchieri et al., 2008), with most homologs exhibiting >40% pairwise identity within their ATPase domains. Evolutionary analysis of the hsp70 family also indicates that mitochondrial Hsp70 proteins are more closely related to bacterial DnaK than to paralogs found in eukaryotes (Deocaris et al., 2006). A comparison of the prototypical DnaK with yeast and human mitochondrial hsp70 chaperones reveals that these proteins exhibit 60% overall amino acid sequence identity (Brocchieri et al., 2008; Craig et al., 1989), with identities as high as 67% in their ATPase domains.

1.2.4. The prototypical DnaK, DnaJ, and GrpE system in *E. coli*.

Much of our understanding of hsp70 chaperone reaction cycles has arisen from biochemical studies of *E. coli* DnaK (for review, *see* Genevoux et al., 2007). As illustrated in Figure 1.1, substrate binding to DnaK is regulated by the nucleotide occupancy of its ATPase domain. In the absence of auxiliary proteins, DnaK is a poor catalyst, exhibiting a slow ATP hydrolysis rate ($<1 \text{ min}^{-1}$) that prevents the futile cycling of ATP (Russell et al., 1998). In the presence of cellular levels of ATP, DnaK associates with ATP and forms the low peptide affinity T state (DnaK-ATP). Cochaperones like DnaJ bind chaperone substrates and direct them to this ATP-bound conformational state (*see* Figure 1.3). Formation of the DnaJ-peptide-DnaK ternary complex leads to a dramatic stimulation of DnaK ATPase activity, up to 10^3 -fold (Karzai and McMacken, 1996), and formation of the high-peptide affinity R-state of DnaK with peptide bound. This stimulation requires the simultaneous binding of the DnaJ J-domain to the DnaK ATPase domain and substrate binding to the DnaK PBD (Karzai and McMacken, 1996; Laufen et al., 1999; Liberek et al., 1991). Because the rate of ADP release by DnaK is slow ($<1 \text{ min}^{-1}$) in the time scale of physiological processes, like ATP hydrolysis (Brehmer et al., 2001), regeneration of the low peptide affinity T-state requires an additional cochaperone, the nucleotide exchange factor GrpE. This cochaperone binds directly to the ATPase domain and stimulates ADP release (Brehmer et al., 2001; Harrison et al., 1997). GrpE is constitutively expressed in *E. coli*, like DnaK and DnaJ. However, under stress conditions where DnaK levels increase, GrpE appears to undergo a reversible conformational change that decreases its activity as a nucleotide-exchange factor. This causes DnaK to form a stable complex with peptides, preventing denaturation

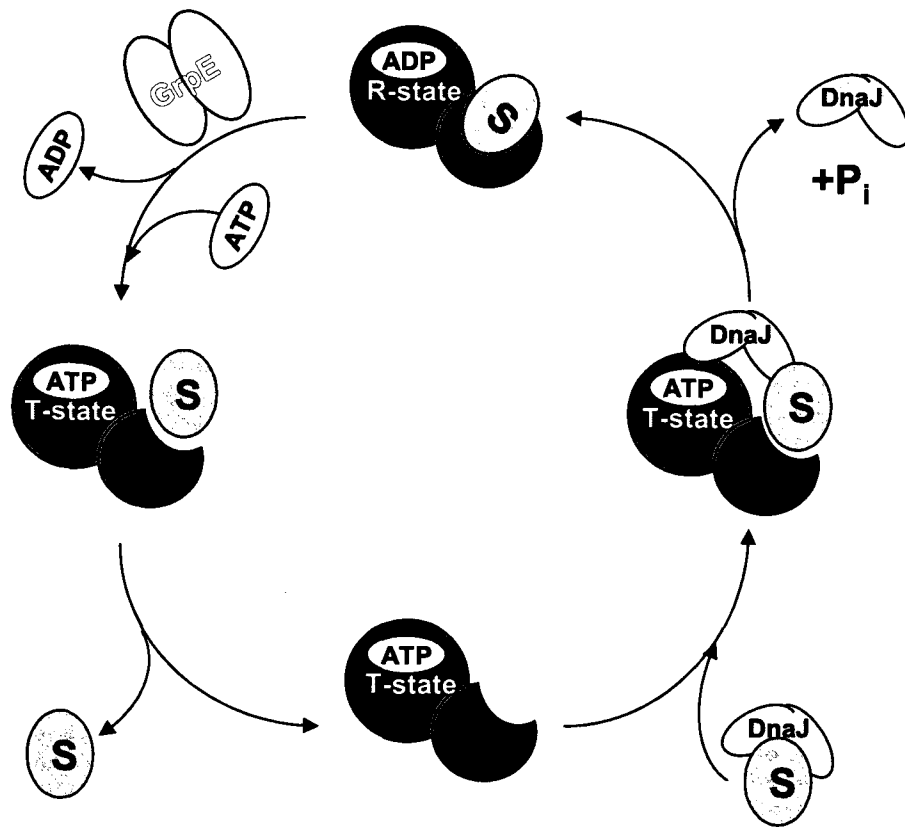


Figure 1.3. Cochaperone regulation of DnaK. DnaJ binds peptide substrates and targets them to ATP-bound DnaK using its N-terminal J-domain. Formation of the ternary DnaK-DnaJ-peptide complex leads to ATP hydrolysis and formation of the DnaK-substrate complex. Substrate release from DnaK requires GrpE, which stimulates ADP release, and allows ATP binding to occur.

and aggregation (Siegenthaler and Christen, 2005; Siegenthaler and Christen, 2006), until the heat shock ends and GrpE becomes active again.

Many DnaJ and GrpE homologs are found in eukaryotes (Qiu et al., 2006), and they are thought to have similar functions as their bacterial homologs. However, the functions of many of these eukaryotic cochaperones remain poorly characterized because many of their cognate chaperones cannot be produced in high levels for *in vitro* biochemical and biophysical studies. For example, human mitochondria contain multiple GrpE homologs (GrpE1 and GrpE2) that are both expressed in diverse tissues (Naylor et al., 1998), but whose exact cellular functions are not yet known. It remains unclear whether these proteins differ in their regulation of mitochondrial hsp70 function.

1.2.5. Hsp70 proteins in yeast and metazoans.

Genomic sequencing has revealed that there are at least ten Hsp70 family members in *Saccharomyces cerevisiae* (Baumann et al., 2000; Werner-Washburne and Craig, 1989). Six of these proteins are predominantly localized to the cytosol, including the two subfamilies Ssa (Ssa1, Ssa2, Ssa3, and Ssa4) and Ssb (Ssb1 and Ssb2), although they can also reside in the nucleus (Werner-Washburne and Craig, 1989). The four other yeast hsp70 family members are enriched in mitochondria (Ssc1, Ssq1 and ECM10) and endoplasmatic reticulum (BiP) (Voos and Rottgers, 2002; Werner-Washburne and Craig, 1989). In some cases, colocalized Hsp70 family members show overlapping functions. For example, deletion of the individual cytosolic Ssa homologs in yeast does not lead to dramatic growth defects, while yeast having mutations in genes encoding multiple hsp70 homologs (*e.g.*, Ssa1, Ssa2 and Ssa4) are not viable (Werner-Washburne et al., 1987). In

other cases, hsp70 family members exhibit distinct non-overlapping functions. Deletion of the predominant yeast mitochondrial Hsp70 (Ssc1) is lethal under any conditions (Craig et al., 1987). In contrast, deletion of another mitochondria Hsp70 isoform, Ssq1, leads to a cold-sensitive phenotype (Schilke et al., 1996). Genomic studies have identified 47 possible Hsp70 sequences in humans that could in theory be generated through alternative splicing (Brocchieri et al., 2008). Mouse knock-out studies have provided evidence that some of the mammalian cytosolic Hsp70 chaperones share overlapping functions (*e.g.*, Hsp70-1a and 1b), as that observed with homologs in yeast (Daugaard et al., 2007). However, it appears that only one Hsp70 homolog is found in mammalian mitochondria. This protein is thought to fulfill the cellular roles of both yeast Ssc1 and Ssq1 (Bhattacharyya et al., 1995).

1.3. Functions of yeast mitochondrial Hsp70 chaperones.

Most of our knowledge about mitochondrial Hsp70 function has come from genetic studies in yeast (Figure 1.4). As noted above, yeast mitochondria contain three Hsp70 family members: Ssc1 (Craig et al., 1987), Ssq1 (Schilke et al., 1996), and Ecm10 (Baumann et al., 2000). Ssc1 is the most abundant isoform and is an essential gene (Craig et al., 1987; Craig et al., 1989). Ssc1 is thought to function as a chaperone for the mitochondrial proteome (Deocaris et al., 2006), preventing protein aggregation. In addition, Ssc1 is indispensable for the translocation of nuclear-encoded proteins into mitochondrial matrix (Neupert and Brunner, 2002). Ssq1 is thought to have a specialized function in the maturation of Fe-S cluster proteins (Vickery and Cupp-Vickery, 2007), like *E. coli* homolog HscA (Chandramouli and Johnson, 2006). The function of Ecm10

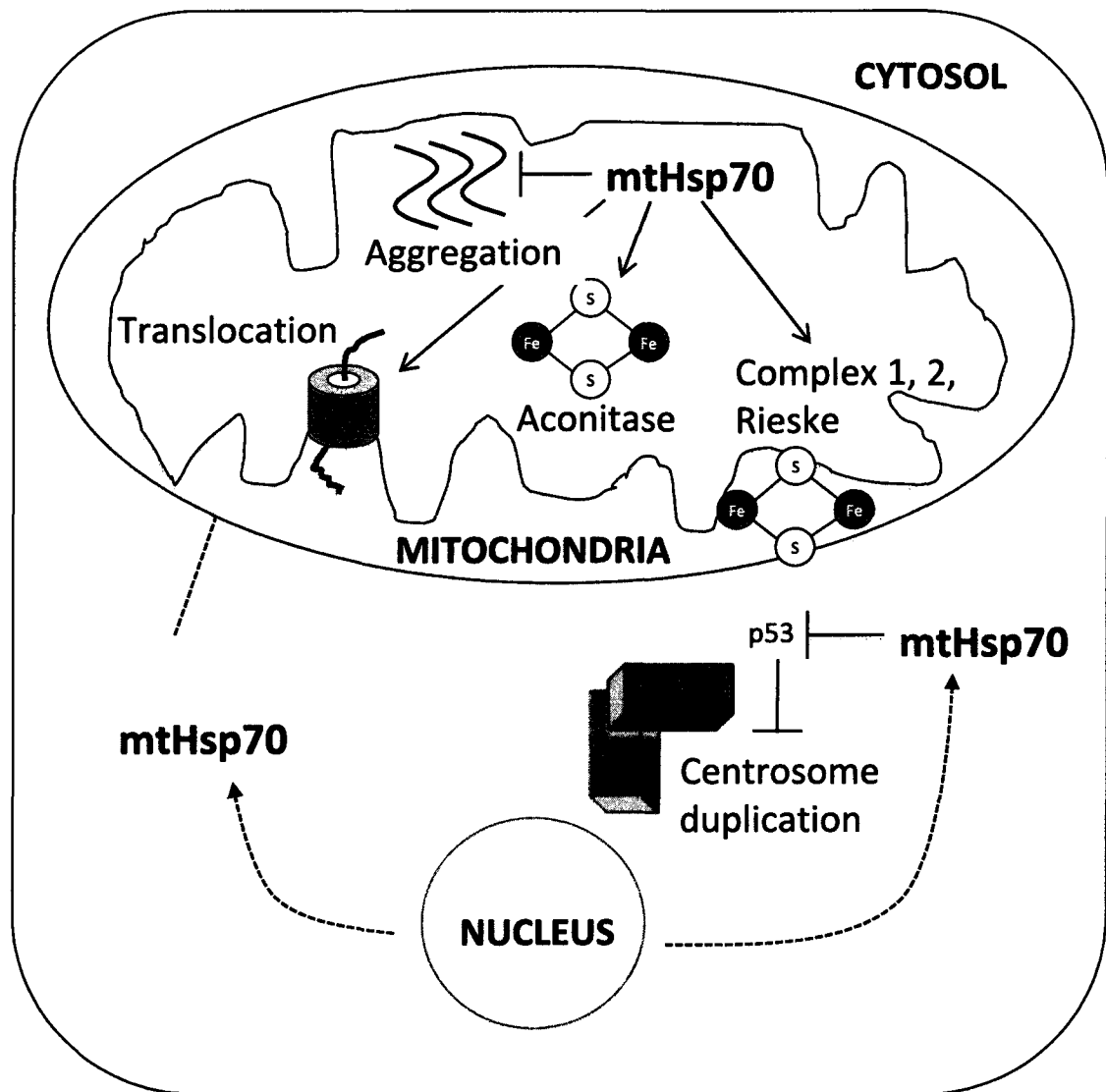


Figure 1.4. Functions of mtHsp70 chaperones. MtHsp70s are nuclear-encoded proteins and are imported into mitochondria after translation in cytosol. In mitochondria, mtHsp70s are essential molecular chaperones for mitochondria proteome. They are also involved in mitochondria protein import and Fe-S-cluster protein maturation. In addition, mtHsp70 in vertebrates are believed to have important roles in cytosol. Human mtHsp70 interacts with p53 and affect the centrosome translocation of p53, which modulates centrosome duplication.

remains unclear. This hsp70 has been proposed to facilitate protein import into mitochondria matrix, similar to Ssc1 (Baumann et al., 2000) . However, it cannot prevent the growth defect of $\Delta ssc1$ yeast (Baumann et al., 2000).

1.3.1. Yeast Ssc1 exhibits classical chaperone activity within mitochondria.

Like the prototypical DnaK chaperone system, Ssc1 functions together with a DnaJ-type cochaperone, Mdj1 (Rowley et al., 1994), and a GrpE-type exchange factor, Mge1 (Miao et al., 1997), as a chaperone system in yeast mitochondria (Voos and Rottgers, 2002). Together, these proteins prevent the aggregation and misfolding of proteins that are newly transported into mitochondrial matrix in a manner similar to that illustrated for DnaK in Figure 1.3 (Horst et al., 1997; Prip-Buus et al., 1996). Ssc1, Mdj1, and Mge1 are also thought to interact with proteins that are newly synthesized by mitochondrial ribosomes, *e.g.* subunits of ATP synthase, preventing their aggregation before they are properly folded and assembled to functional units (Herrmann et al., 1994). Furthermore, Ssc1 has been shown to direct misfolded and aggregated proteins to mitochondrial proteases for degradation (Savel'ev et al., 1998; Wagner et al., 1994).

1.3.2. Yeast Ssc1 is essential for the protein import into mitochondria matrix.

The great majority of mitochondrial-localized proteins are nuclear-encoded and translocated into mitochondria (Herrmann and Neupert, 2000; Wiedemann et al., 2004). Genetic studies have provided evidence that Ssc1 is essential for this process, as yeast strains having a temperature-sensitive Ssc1 cannot translocate proteins into the mitochondrial matrix at the nonpermissive temperatures (Gambill et al., 1993; Neupert

and Brunner, 2002; Voos and Rottgers, 2002). Biochemical studies have shown that a direct interaction is required between Ssc1 and polypeptides that are being translocated, and complete preprotein translocation is thought to require multiple cycles of Ssc1 binding and release, and ATP hydrolysis (Glick, 1995; Glick et al., 1993). Two different models have been proposed to explain the need for Ssc1 during protein translocation (Neupert and Brunner, 2002). First, Ssc1 has been proposed to act as a molecular clamp that prevents the backward movement of preproteins that are partially translocated into the mitochondrial matrix (Neupert et al., 1990; Schneider et al., 1994; Simon and Blobel, 1993; Simon et al., 1992). In this model, Ssc1 acts as a ratchet preventing the backwards movement of preproteins, and Brownian motion drives the forward movement (Schneider et al., 1994). Second, Ssc1 has been proposed to function as a motor that actively pulls the preprotein into the matrix (Neupert and Brunner, 2002).

Ssc1 is thought to use a reaction cycle similar to that described for DnaK to aid in protein translocation (Neupert and Brunner, 2002; Voos and Rottgers, 2002). The inner membrane-associated protein Tim44 is a J-domain protein that sits on the outlet end of Tim23 channel (Moro et al., 1999) and recruits only ATP-bound Ssc1 to the import channel (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). Once Ssc1 is recruited to the membrane, it forms a ternary complex with another J-domain protein (Pam18) and preproteins being translocated (D'Silva et al., 2003). Formation of this ternary complex is thought to stimulate ATP hydrolysis by Ssc1 and formation of a high affinity Ssc1-preprotein complex (D'Silva et al., 2003). This Ssc1-preprotein complex is thought to dissociate from Tim44 thereby allowing the preprotein chain to move further into mitochondria matrix but not back into the cytosol (Schneider et al., 1994;

Ungermann et al., 1996). At this stage, the GrpE-type exchange factor Mge1 is thought to bind to Ssc1 and stimulate nucleotide-exchange and dissociation of the preprotein substrate (Laloraya et al., 1995; Laloraya et al., 1994; Schneider et al., 1996; Voos et al., 1994; Westermann et al., 1995).

1.3.3. Ssq1 is needed for mitochondrial iron-sulfur cluster biogenesis.

Fe-S clusters are essential cofactors for proteins with roles in catalysis, electron transfer, and regulation (Muhlenhoff and Lill, 2000; Schilke et al., 1999). Studies have shown that one of the minor Hsp70 homologs in the mitochondria (Ssq1) is needed for normal Fe-S cluster biogenesis in yeast mitochondria (for reviews, *see* Vickery and Cupp-Vickery, 2007; Voos and Rottgers, 2002). Ssq1 is not an essential gene, indicating that its function can be partially substituted by Ssc1 (Voisine et al., 2000). Deletion mutants of Ssq1 result in mitochondrial DNA loss and a cold-sensitive growth defect (Schilke et al., 1996). Iron accumulation and reduced activity of Fe-S cluster enzymes are also observed (Knight et al., 1998; Lutz et al., 2001). Moreover, Ssq1 directly binds to the Fe-S cluster scaffold protein Isu in yeast mitochondria, which is an iron-sulfur cluster template protein where Fe-S clusters are initially synthesized in aerobic organisms (Dutkiewicz et al., 2004). Isu is thought to behave as a substrate for Ssq1, as studies with bacterial homologs have shown that the PBD binds a conserved LPPVK motif (Hoff et al., 2002). Ssq1 binding to Isu is regulated by the small J-like cochaperone Jac1 (*J*-type *a*ccessory *c*haperone), a protein that contains a N-terminal J-domain similar to the domain in DnaJ that binds DnaK, and a C-terminal domain that binds Isu (Schilke et al., 2006). The gene encoding Jac1 is essential (Strain et al., 1998), and a conditional deletion

of its gene results in decreased activity of Fe-S cluster dependent enzymes (Kim et al., 2001; Muhlenhoff et al., 2003; Voisine et al., 2001). *In vitro* studies with *Azotobacter vinlandii* and *E. coli* homologs of this specialized Ssq1/Jac1/Isu system (the bacterial HscA/HscB/IscU system) have shown that Hsp70 chaperones are needed to catalyze the transfer of 2Fe2S clusters initially built on IscU to apoacceptors like ferredoxins (Bonomi et al., 2008; Chandramouli and Johnson, 2006). Most multicellular eukaryotes lack a Ssq1-type chaperone, although they invariably have Ssc1 chaperone. This finding has suggested that the Jac1-type cochaperones found in higher eukaryotes like mammals have evolved to target Isu-type proteins to Ssc1 orthologs (Schilke et al., 2006).

1.4. Functions of metazoan mitochondrial Hsp70 chaperones.

Unlike yeast, which have multiple hsp70 chaperones in their mitochondria, vertebrates are thought to have only a single mitochondrial hsp70 family member (Bhattacharyya et al., 1995). In humans, a number of names are used with this protein, including mortalin, HspA9b, and mtHsp70. Human mtHsp70 is thought to be essential (Daugaard et al., 2007; Kaul et al., 2002), like yeast Ssc1 (Craig et al., 1987; Craig et al., 1989), and sequence alignments indicate that mtHsp70 is most closely related to yeast Ssc1. Human mtHsp70 exhibits 62% identity with Ssc1 and only 45% identity with Ssq1. Human mtHsp70 is believed to fulfill the functions carried out by yeast Ssc1 and Ssq1 described above (Kaul et al., 2002). In addition, human mtHsp70 is thought to have additional cellular roles in cell proliferation, differentiation, and tumorigenesis (for review, *see* Kaul et al., 2002). Although mtHsp70 appears to have more functions than Ssc1 and Ssq1, most of our insight into the structure, function, and regulation of this chaperone has come from

studies of distantly-related homologs (Genevaux et al., 2007; Voos and Rottgers, 2002). This has occurred because recombinant human mtHsp70 could not be generated in a soluble functional form until the studies described in my thesis.

1.4.1. mtHsp70 is subject to post-translational modifications.

Human mtHsp70 has been shown to be subject to multiple post-translational modifications, which are expected to have effects on its activities. In the cytosol, mtHsp70 has been shown to interact with the kinase Mps1 and shown to be phosphorylated by Mps1 at Thr62 and Ser65 (Kanai et al., 2007). This phosphorylation has been shown to have effects on Mps1 activity, but its effect on mtHsp70 activities remains unclear. A proteomic study screening the mitochondrial proteome for acetylated proteins identified three lysine residues in mtHsp70 as having this modification (Kim et al., 2006). All three residues lie near each other within the mtHsp70 ATPase domain, but their role in regulating mtHsp70 function also remains unknown. The acetylation and phosphorylation state of mtHsp70 may regulate its intrinsic catalytic activity, binding to substrates, or interactions with cochaperones.

1.4.2. Differences in human and yeast mitochondrial cochaperones.

Cochaperone regulation of human mtHsp70 activity may be more complex than that observed for yeast homologs, as human mitochondria contain more cochaperones than fungi. For example, fungi only have a single GrpE-type exchange factor, Mge1 (Laloraya et al., 1995; Laloraya et al., 1994; Schneider et al., 1996; Voos et al., 1994; Westermann et al., 1995), whereas two isoforms (GrpE1 and GrpE2) have been identified in

vertebrates (Naylor et al., 1998). The functions of these two closely related isoforms have not yet been distinguished, although a biophysical study showed that GrpE1 exhibits a higher resistance to thermal and chemical denaturation than the GrpE2 (Oliveira et al., 2006). In addition, there are differences between the human and fungal J proteins that regulate substrate binding. Homologs of the yeast J-proteins involved in protein folding (Mdj1), translocation (Tim44 and Pam18) and Fe-S cluster biogenesis (Jac1) are found in humans (Bauer et al., 1999; Elsner et al., 2009; Neupert and Brunner, 2002) (Sun et al., 2003). However, human Jac1 (termed HscB in mammals) has a domain that is not found in any fungal homologs of this protein. All bacterial and fungal HscB homologs characterized to date contain a J-domain for binding to cognate Hsp70 chaperone (Greene et al., 1998) and a C-terminal domains to bind their only know substrate, IscU (Silberg et al., 2001; Silver and Erecinska, 1998). However, sequence analysis of human HscB (Sun et al., 2003) shows it has an addition cysteine-rich domain at their N-terminus with unknown function. A similar domain structure is also found in HscB homologs from other vertebrates, plants, protozoans, and < 1% of bacteria. To date, no fungi HscB homologs have been found that contain this domain structure (Bitto et al., 2008). This cysteine-rich domain is characterized by the presence of a tetracysteine motif (CWxCx₈-₁₃CxxCx₃Q), which may coordinate divalent metal ions and inorganic iron-sulfur clusters (Beinert et al., 1997). The physiological cofactor for this domain is not known, although a recent structure of human HscB found that Zn⁺² was coordinated by this novel domain (Bitto et al., 2008).

1.4.3. Human mtHsp70 has cytosolic functions.

Unlike Ssc1 and Ssq1, which are predominantly found within the mitochondria, mtHsp70 can be found in both the cytosol and mitochondria (Wadhwa et al., 1995), with increased cytosolic expression observed in cancer cells lines (Czarnecka et al., 2006; Wadhwa et al., 2006; Yi et al., 2008). The exact cytosolic role of mtHsp70 is not well understood. However, human mtHsp70 has been shown to affect centrosome duplication by modulating the centrosomal localization of p53 (Figure 1.4) (Ma et al., 2006) and Mps1 kinase (Kanai et al., 2007). *In vitro* and in tissue culture studies have provided evidence that mtHsp70 binds to the C-terminal cytoplasmic sequestration sequence of p53 using its ATPase domain (Kaul et al., 2001; Wadhwa et al., 2002). This interaction has been shown to inactivate p53 by preventing its nuclear translocation (Wadhwa et al., 1998). Whether mtHsp70 requires cochaperones to regulate its binding to p53 is not known.

1.5. Human mtHsp70 and human diseases.

1.5.1. A mutation in zebrafish mtHsp70 causes a blood development defect.

Myelodysplastic syndrome (MDS) is characterized by a group of heterogeneous blood disorders including anemia, dysplasia, increased apoptosis of blood cells, and multilineage cytopenia (Kurzrock, 2002; Vergilio and Bagg, 2003). In human, there is evidence that MDS is caused by deletion of a region within chromosome 5q31 that contains the gene encoding mtHsp70 (Horrigan et al., 2000; Liu et al., 2002). In *Danio rerio*, a mutant called *crimsonless* (*crs*) has also been discovered that causes a phenotype similar to that observed in patients with myelodysplastic syndrome (MDS) (Craven et al.,

2005). In this study, two overlapping P1-derived artificial chromosome (PAC) clones containing the complete coding sequence for zebrafish mtHsp70 were capable of rescuing the anemia in *crimsonless* zebrafish (Craven et al., 2005). DNA sequencing revealed that the mtHsp70 in *crs* has a single glycine-to-glutamate substitution at position 492 (G492E), which is within its peptide-binding domain (Craven et al., 2005). A similar mutation in *E. coli* DnaK (G443D) has been shown to completely eliminate its substrate binding activity (Burkholder et al., 1996), suggesting a possible molecular basis for the phenotype observed. Furthermore, injection of capped RNA encoding wild-type mtHsp70 rescued the defects in ~95% of *crimsonless* embryos (Craven et al., 2005).

1.5.2. Changes in mtHsp70 expression are correlated with multiple diseases.

In mammals, a correlation between cell viability and changes in mtHsp70 expression has been observed. Increased expression of human mtHsp70 has been suggested to represent a marker for tumors with greater malignancy and metastasis in colon, leukemia, and liver cancers (Czarnecka et al., 2006; Wadhwa et al., 2006; Yi et al., 2008). A recent clinical study on hepatocellular carcinoma has provided evidence that the abundance of mtHsp70 in cancerous cells may be the best marker for predicting the probability that there will be an early recurrence of that cancer after surgical removal of the primary tumor (Yi et al., 2008). These overexpression phenotypes are thought to arise at least in part because mtHsp70 overrides the p53-dependent suppression of centrosome duplication in the cytosol (Bottger et al., 2008; Kaul et al., 2005; Ma et al., 2006). Efforts are currently underway to develop novel chemotherapeutics to disrupt mtHsp70 binding to p53 (Bottger et al., 2008; Kaul et al., 2005).

Increased expression of mtHsp70 has also been correlated with extended lifespan in human fibroblast cells (Kaul et al., 2003) and *Caenorhabditis elegans* (Yokoyama et al., 2002). Overexpression of mtHsp70 alone can extend human fibroblast lifespan up to 15 to 20% (Kaul et al., 2003; Kaul et al., 2000). In addition, mtHsp70 overexpression can work cooperatively with telomerase (which increases lifespan by <10% upon overexpression) and extend the lifespan of human fibroblasts up to ~100% (Kaul et al., 2003). Similar findings have been reported with *C. elegans* mtHsp70. Unlike the mammalian counterparts, the mtHsp70 ortholog in *C. elegans*, Hsp-6 (also termed as Hsp70F), is a heat-shock protein. Transgenic worms carrying an extra copy of Hsp-6 display a >40% extended lifespan (Yokoyama et al., 2002). In contrast, decrease of the expression level of mtHsp70 may lead to an opposite effect cell growth. Studies of *C. elegans* have reported that the Hsp-6 level is frequently decreased in aged organisms, and they have found that knockdown of the gene encoding Hsp-6 by RNA interference in young adults causes progeria-like phenotypes, including lower mobility, defective oogenesis, early accumulation of autofluorescence material, and shortened lifespan (Kimura et al., 2007).

Lower expression of mtHsp70 may also be correlated with the progression of neurodegeneration, like Parkinson's disease (PD) (Jin et al., 2006; Shi et al., 2008). A shotgun proteomics study found that the level of mtHsp70 in mitochondria is dramatically decreased in the brains of PD patients and in cell models of PD (Jin et al., 2006). This decrease may be caused by the failure of transporting mtHsp70 into mitochondria as the total cellular mtHsp70 level did not change significantly in the PD cell model (Jin et al., 2006). A severe decrease in mtHsp70 levels may lead to Lewy body

deposition (the pathological feature of PD) and progression of PD (Jin et al., 2006; Shi et al., 2008). Furthermore, mtHsp70 overexpression protects PD model cells against rotenone treatment, a mitochondria complex I inhibitor that induces cellular damage (Betarbet et al., 2000), while the down regulation of mtHsp70 produced the opposite effects (Jin et al., 2006).

1.6. Mitochondrial Hsp70 Escort Proteins.

1.6.1. Discovery of the Hsp70 Escort Protein Hep1.

The hsp70 escort protein Hep1 (also termed Zim17 for 17-kDa Zinc-finger motif protein, Tim15 for 15-Kd Trans-inner membrane protein, and YNL310c) was initially discovered in a bioinformatic study that was screening the *S. cerevisiae* genome for genes that encode domains related to the zinc-finger domain found in the *E. coli* cochaperone DnaJ (Burri et al., 2004). Besides five previously known J-proteins, two additional proteins (Hua and Hep1) were identified in this screen, which had no other known domain signatures (Burri et al., 2004). A previous study had implicated a role for Hua in the assembly and disassembly of actin cytoskeleton (Samanta and Liang, 2003), whereas Hep1 was a predicted mitochondria protein (Sickmann et al., 2003) with no known functions. Based on sequence comparisons with J-type cochaperones, yeast Hep1 was proposed to represent a “fractured” J-protein because it lacks an N-terminal J-domain found in DnaJ homologs but contains a tetracysteine motif like DnaJ. Hep1 was found to be mitochondrial localized using an *in vitro* protein import assay (Burri et al., 2004), and it was shown to be important for the import of other proteins into the mitochondrial matrix (Burri et al., 2004; Yamamoto et al., 2005). In a subsequent study, Hep1 was

identified as a protein that copurifies from *S. cerevisiae* mitochondria lysate with His-tagged mtHsp70. This study provided the first evidence that Hep1 and mtHsp70 may function together, and it demonstrated that the Hep1-mtHsp70 complex is destabilized by ATP (Sichting et al., 2005). Although there is now clear evidence that yeast Hep1 is a nuclear-encoded protein that is localized to the mitochondria (Burri et al., 2004; Sichting et al., 2005), its sub-organellar localization remains unclear. It is widely believed that Hep1 is a mitochondrial matrix protein that loosely associates with the inner membrane via ionic interactions (Burri et al., 2004; Sanjuan Szklarz et al., 2005; Yamamoto et al., 2005). This localization is thought to enable Hep1 to regulate mtHsp70 as it functions in translocating nuclear-encoded proteins into the mitochondria matrix (Burri et al., 2004; Yamamoto et al., 2005), although this has not yet been directly demonstrated.

Quantitative immunoblotting has provided evidence that Hep1 is present in mitochondria at a concentration that is ~5% of the level of Ssc1 and Ssq1 (Sichting et al., 2005). This finding suggested that Hep1 can form at most a stoichiometric complex with a small fraction of the mitochondrial Hsp70 chaperones at any one time (Sichting et al., 2005). However, there is evidence that Hep1 is a heat shock protein. A heat-shock element (HSE)-like sequence similar to other mitochondrial chaperones (Tachibana et al., 2002) is present in the 5'-untranslated region of yeast Hep1 (Sichting et al., 2005), and Hep1 exhibits elevated expression after heat treatment (Sanjuan Szklarz et al., 2005).

1.6.2. Hep1 depletion causes chaperone-like defects.

Hep1 conditional knock out strains exhibit mitochondrial defects consistent with Ssc1 and Ssq1 depletion. These include decreased import of nuclear-encoded proteins into

mitochondria matrix (Burri et al., 2004; Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Yamamoto et al., 2005), impaired Fe-S cluster biogenesis (Sanjuan Szklarz et al., 2005; Sichting et al., 2005), and pleiotropic effects on mitochondrial morphology (Sanjuan Szklarz et al., 2005). Multiple studies have shown that Hep1 deletion selectively decreases protein import mediated by Tim23 complex (Burri et al., 2004; Sichting et al., 2005). However, Hep1 depletion does not disrupt the assembly of the Tim23 complex, and Hep1 itself does not appear to be a component in the Tim23 complex in wild-type yeast (Burri et al., 2004; Sichting et al., 2005). Hep1 depletion also causes a decrease in the activities of Fe-S enzymes, including those involved in the citric acid cycle (aconitase) (Sanjuan Szklarz et al., 2005) and respiration (succinate dehydrogenase) (Sichting et al., 2005). In addition, the mitochondria of yeast that have a temperature-sensitive Hep1 (*zim17-2*) become highly fragmented after growth at elevated temperatures overnight; this change is not seen upon a short (4 hour) heat treatment (Sanjuan Szklarz et al., 2005).

Multiple studies have observed the aggregation of Hsp70 chaperones in yeast strains having decreased levels of the escort protein Hep1 (Sanjuan Szklarz et al., 2005; Sichting et al., 2005). In addition, *E. coli* expression studies have shown that the yeast chaperone Ssc1 is insoluble unless it is coexpressed with Hep1 (Sichting et al., 2005). Similar studies in *Chlamydomonas* have found that the plastid Hsp70 molecular chaperone, Hsp70B, requires an escort protein (Hep2) for solubility (Willmund et al., 2008). These findings suggest that Hep1-type escort proteins may be needed to regulate the solubility of both mitochondrial and plastid Hsp70 chaperones. Escort protein regulation of chaperone solubility appears to involve the *inhibition* of chaperone aggregation, as studies

examining the addition of yeast Hep1 to aggregated mtHsp70 has no effect on chaperone solubility (Sichting et al., 2005).

Genetic studies examining the effect of Hep1 overexpression on the growth of yeast strains with mutations in the genes encoding their mtHsp70 chaperones suggest that Hep1 may interact directly with the ATPase domain (Yamamoto et al., 2005). Overexpression of Hep1 complements the growth of yeast expressing a Ssc1 variant (*ssc1-3*) that has a mutation in the ATPase domain (Gambill et al., 1993), while it has no effect on the growth of a strain that expresses an Ssc1 variant (*ssc1-2*) with a mutation in the PBD (Gambill et al., 1993).

1.6.3. Evolutionary conservation of escort proteins.

As shown in Figure 1.5, Hep orthologs are found in most eukaryotes, including fungi, coelenterates, worms, protozoan, insects, plants, and higher vertebrates. In addition, distinct homologs are found in the mitochondria (Burri et al., 2004; Sichting et al., 2005) and plastids (Willmund et al., 2008) of eukaryotes, which are of similar size (~200 residues). However, no Hep homologs have been discovered in prokaryotes, suggesting that these proteins evolved after the endosymbiosis of bacteria into mitochondria (Burri et al., 2004; Yamamoto et al., 2005). A comparison of Hep1 and Hep2 homologs indicates that all family members contain a zf-DNL zinc finger domain, defined in the “Pfam Protein Families Database” (Bateman et al., 2004), a predicted N-terminal organellar targeting sequences, and a negatively charged region at the C-terminal which is poorly conserved (Burri et al., 2004). While the Hep sequences from different species are not highly conserved, typically <30% overall pairwise identity, the tetracysteine motif is

```

Hs      -----MLRTALRGAPR-----LLSRVQPRAPCLR
Mm      -----MLRTALSRMPT-----LLRSVTRDMSGPR
Ce      -----MLRSVSAVS-----A-----LYRASRSFS-----
Zm      -----MATTRLPLLRRLAAAIAG-----SPAPYSLRGPSFPAPAAAG--LR
Os      -----MAT-REFLPLVRRGLAGVLNQ-----SPAPASTRGFLFPAPVTAG--IR
At      -----MAA-RLI-ALRRALSLSFSNQHRFPLSQVSTEQLSLNSLFSRSHVYGRLFQ
Rn      -----MLRTALSRMPT-----LLRSVTRDMSGPR
Cr      MLQTKSWAAQAGAGVGRAFSARLGCPTLGRTPSPLLSTTKASTYTTSSGTYGLASSSSGFGAR
Nc      -----MASKRAAASTLSCIIRLGSAPAQSLKTTTTTSRFLNYFVHSYSSQCQRPVPLSPQ
Sc      -----MIPRTRT-----LLQSKIPITRYFA

Hs      RLWGRGARPEVAGRRRAWAWG---WRRSSSEQGPGP-----AAALGRVEAAHYQLV
Mm      RLWDLGARLKTAERLRGAWGWASGWRSSSSAPGSGR-----AAALGRVEADHYQLV
Ce      -----ETVPRLS-----ATPLGKQEP-QLSL
Zm      SLLTVAGASDTATEP--QDQHSSETTPPPASVPTPESG--LKVRDTSNLKISPRHDLAMI
Os      SLQTIMEASNNASDDRNQDIEDSKDTVPATVPSSDSG--FKVRDTSNLKISPRHDLAMI
At      RQLSVIREANEASVTNVCNSSNSATESAKVPSPATPSEE--MMVKYKSQLKINPRHDFMMV
Rn      QLWDLGARLKTAKRLRGAWG---WRSSSSAPGSEH-----AAALGRVKADHYQLV
Cr      VQQLPRWACRATVDPIIPAGLQIEVKEEDGTKEVDYDEEKKVVRIPLSAMGDGARRSKLVL
Nc      QQTQQRGDIINYTPIRQKHTIPRPRSQFSSSPEFQSPFSD--QQQPAQPKRDTSQVPQYELT
Sc      RCWAPRVRYNVCRTLPAALHTNI IAHNEVKKDDKKVH-----LGSFKVDKPKMMIA

Hs      YTCVKCGTR-SSKRI SKLAYHQGVVIVTCPGCQNHIIADNLGWFSDLNGKRNIEEILTA
Mm      YTCVKCGTR-SSKRI SKLAYHQGVVIVTCPGCQNHIIADNLGWFSDLNGKRNIEEILAA
Ce      YTCVKNREGPKTFKSSYEKGVVIVTCGCHNHIIADNLGWFEDFKGK-NIEDHLKT
Zm      FTCKVCETR-SMKMASRDSYENGVVVVRCGGCNNLHLMADRLGWFGEPG---SIEDFLAT
Os      FTCKVCETR-SMKMASRDSYENGVVVVRCGGCNNLHLMADRLGWFGEPG---SIEDFLAE
At      FTCKVCETR-SMKMASRDSYENGVVVVRCGGCNNLHLMADRLGWFGEPG---SIEDFLAS
Rn      YTCVKCGTR-SSKRI SKLAYHQGVVIVTCPGCQNHIIADNLGWFSDLNGKRNIEEILAA
Cr      FTCKKCGGR-SARLVNPVAWEKGVVFGCCSKCSVWHVLQANNKKIYEEVRYNEDPEYADK
Nc      FTCKPCDHR-SKHKVSQGYHHGVSVLISCPNCRNRHVI SDHLKLFGRK---VTVEDLLKE
Sc      FTCKKCNTR-SSHIMSKQAYEKGTVLISCPHCKVRHLIADHLKIFHDH---VTVEQLMKA
: ** * * . : . : * * . * * * : . : :

Hs      RGEQVHRVAG-----EGALELVLEAAGAPTSTAAPAGEDEGPPSPGKTEPS-----
Mm      RGEVRRVSG-----DGALELILEAAVFP---DTPEGDED--PPNPGKMGQS-----
Ce      RGEAVKRRDT-----IKNENGIFEIQK-----PPNPGKMGQS-----
Zm      QGEEVKKGST-----DTISFTLDDLAGSQVSSKGPSEQN-----
Os      QGEEVKKGST-----DTLNFTLEDLVGSQANDKGPSDKK-----
At      QGEEFKKGS-----DSLNLTPEDIAGKISTE-----
Rn      RGEVRRVCG-----DGALELILEVAEPF---ATPEGGED--PTHFGKMDQS-----
Nc      KGMVVRGTLGEGDGVFWDGTSTMREPEPEPAPERPKKEVDNSFPGSTTFKNVRPGEKKDGDES
Cr      DGLDAK-----AEELLKQAVAAEAEPNKQ-----
Sc      NGEQVSQDVG-----DLEFEDIPDSLKDVLGKYAKNNSENASQLPHPSQK-----
* :

```

Figure 1.5. Sequence alignment of escort proteins. The alignment was generated using the SDSC Biology workbench (<http://workbench.sdsc.edu>) accepting all default values. The sequences shown include escort protein homologs from *Saccharomyces cerevisiae* (Sc), *Chlamydomonas reinhardtii* (Cr), *Neurospora crassa* (Nc), *Rattus norvegicus* (Rn), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm), *Caenorhabditis elegans* (Ce), *Mus musculus* (Mm), and *Homo sapiens* (Hs). Identical residues are indicated with an asterisk, highly similar are indicated with a colon, and similar are indicated with a period. In addition, the four conserved cysteine residues were highlighted in black.

absolutely conserved in all homologs. In addition, a handful of residues are absolutely conserved across all species, which correspond to the following residues in yeast Hep1: threonine 74, arginine 81, glycine 94, valine 96, histidine 107, and glycine 131.

1.6.4. Structural studies of *S. cerevisiae* Hep1.

The structure of the trypsin-resistant domain of yeast Hep1 (Figure 1.6) was determined under non-physiological conditions (pH = 4.5) using NMR spectroscopy (Momose et al., 2007). This structure shows that Hep1 is not a structural homolog of *E. coli* cochaperone DnaJ, as previously proposed (Martinez-Yamout et al., 2000). In addition, this structure (residues 64 to 159) shows that the four cysteine residues conserved in all escort proteins are located on proximal two-stranded antiparallel β -sheets and oriented in a manner that is consistent with the coordination of a metal ion (Momose et al., 2007). The binding of Zn^{2+} was found to be essential for amide resonance dispersion in the HSQC spectrum, implicating zinc as the metal bound by this tetracysteine motif (Momose et al., 2007). Zinc binding to Hep1 is thought to be essential for escort protein function. Hep1 mutants harboring either a C75S or C100S mutation do not rescue the growth defects of a Δhep1 yeast (Yamamoto et al., 2005). Whether the other two cysteines in the motif are needed for escort protein function is not known. Furthermore, it is not known if the NMR structure determined for Hep1 at pH 4.5 accurately reflects the structure of Hep1 in yeast mitochondria with a resting pH around 8.0 (Llopis et al., 1998).

Several Hep1 mutants have been made based on its structure, and the function of these mutants have been characterized *in vivo* (Momose et al., 2007). These experiments

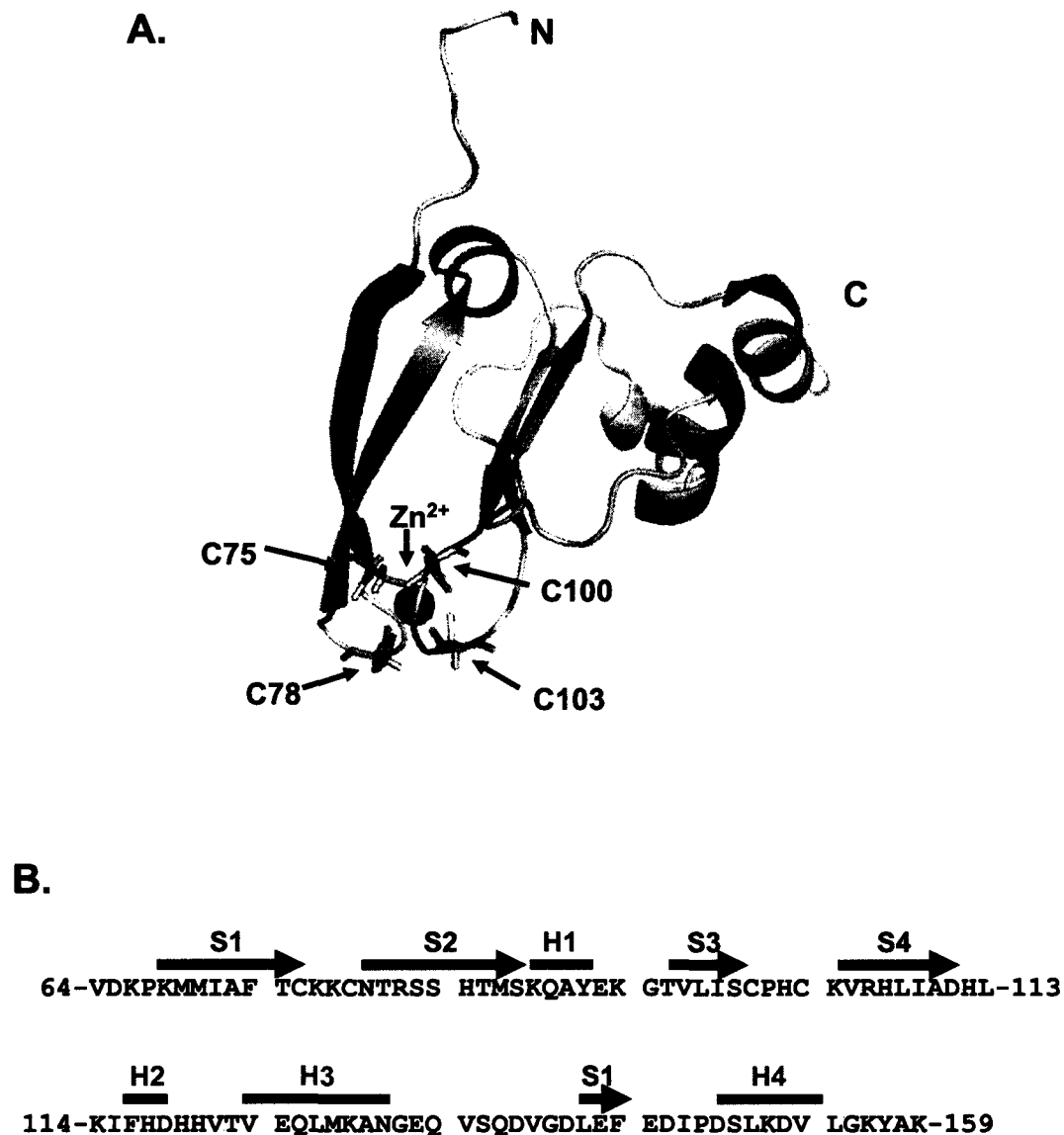


Figure 1.6. NMR structure of the trypsin-resistant core domain of Hep1.
A. Ribbon representation of the solution structure of *S. cerevisiae* Hep1 trypsin resistant core-domain (PDB = 2E2Z). The four conserved cysteines and bound Zn^{2+} ion are labeled. **B.** The primary sequence of the Hep1 core domain with the secondary structure elements shown as bars (α helices) and arrows (β sheets).

revealed that two mutants could not complement the growth of $\Delta hep1$ yeast, including a mutant with alanine substitutions at arginine106 and histidine107 (termed as 2RH) and a variant with an alanine substitution at aspartate 111 (D111A). In addition, a mutant with the loop region between α -helix 3 and β -sheet 5 (termed $\Delta 133-137$) deleted could only partially complement $\Delta hep1$ yeast. In contrast, the neutralization of five negatively charged residues on an acidic groove of Hep1 had no effect on protein function.

Aggregation of mtHsp70 was also observed in the mitochondria of yeast cells harboring the 2RH, D111A, and $\Delta 133-137$ Hep1 mutants (Momose et al., 2007). However, no *in vitro* studies were performed to evaluate why these Hep1 mutants exhibit defects *in vivo*, or if these mutants have defects in binding to and regulating the activity of ATP-bound chaperones.

1.7. Summary and overview of my thesis research.

As reviewed above, genetic studies have shown that *S. cerevisiae* lacking a functional Hep1 exhibit deficiencies consistent with mtHsp70 depletion, including decreased import of nuclear-encoded proteins into the mitochondrial matrix, reduced activities of the Fe-S proteins, and pleiotropic effects on mitochondrial morphology (Burri et al., 2004; Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Yamamoto et al., 2005). These phenotypes have been proposed to arise because the mtHsp70s Ssc1 and Ssq1 aggregate in the absence of Hep1 and are unable to fulfill their functions (Sanjuan Szklarz et al., 2005; Sichting et al., 2005). Prior to the studies herein, it was unclear which domain(s) in mitochondrial Hsp70 chaperones were responsible for its poor solubility in the absence of escort proteins (Bhattacharyya et al., 1995), and it was not known if escort proteins

bound to the C-terminal PBD of their cognate chaperones, like peptide substrates (Cupp-Vickery et al., 2004; Zhu et al., 1996), or the N-terminal ATPase domain, like J- (Deloche et al., 1997) and E-type (Brehmer et al., 2001; Harrison et al., 1997) cochaperones. In addition, it was unclear if genes encoding Hep1-like proteins could promote the solubility of metazoan hsp70 chaperones and alter their function, such as the catalytic activity of ATP-bound mtHsp70.

To better understand escort protein regulation of chaperone folding and function, I characterized the solubility of human mtHsp70, its isolated ATPase domain (designated 70^{ATPase}), and human-bacterial hsp70 chimeras to determine the domain that is responsible for mtHsp70 misfolding upon overexpression in bacteria. I also examined the effect of human Hep on the solubility of these proteins, determined the Hep-interacting domain of mtHsp70, characterized the effect of nucleotides on human Hep-mtHsp70 binding, and evaluated the effect of Hep on the ATPase activity of mtHsp70. Furthermore, I investigated whether residues conserved in yeast and human Hep homologs are important for human Hep regulation of mtHsp70 solubility and ATPase activity, and I investigated the effects of human Hep expression on the growth defects of $\Delta hep1$ yeast, as well as a Hep mutant exhibiting *in vitro* defects. Finally, I identified a 59 residue region within human Hep that is necessary and sufficient to regulate Hsp70 solubility.

Chapter 2

The human escort protein Hep binds to the ATPase domain of mitochondrial Hsp70 and regulates ATP hydrolysis

Abstract

Hsp70 escort proteins (Hep) have been implicated as essential for maintaining the function of yeast mitochondrial hsp70 molecular chaperones (mtHsp70), but the role that escort proteins play in regulating mammalian chaperone folding and function has not been established. I present evidence that human mtHsp70 exhibits limited solubility due to aggregation mediated by its ATPase domain and show that human Hep directly enhances chaperone solubility through interactions with this domain. In the absence of Hep, mtHsp70 was insoluble when expressed in *Escherichia coli*, as was its isolated ATPase domain and a chimera having this domain fused to the peptide-binding domain of HscA, a soluble monomeric chaperone. In contrast, these proteins all exhibited increased solubility when expressed in the presence of Hep. *In vitro* studies further revealed that purified Hep regulates the interaction of mtHsp70 with nucleotides. Full-length mtHsp70 exhibited slow intrinsic ATP hydrolysis activity ($6.8 \pm 0.2 \times 10^{-4} \text{ sec}^{-1}$) at 25°C, which was stimulated up to 49-fold by Hep. Hep also stimulated the activity of the isolated ATPase domain, albeit to a lower maximal extent (11.5-fold). In addition, gel filtration studies showed that formation of chaperone-escort protein complexes inhibited mtHsp70 self-association, and they revealed that Hep binding to full-length mtHsp70 and its isolated ATPase domain is strongest in the absence of nucleotides. These findings

provide evidence that metazoan escort proteins regulate the catalytic activity and solubility of their cognate chaperones, and they indicate that both forms of regulation arise from interactions with the mtHsp70 ATPase domain.

2.1. Introduction

The hsp70 protein family is a ubiquitous class of proteins found in most cellular compartments that have evolved to participate in a range of cellular processes, including vesicular trafficking, Fe-S-cluster biogenesis, the stress response, protein folding, and protein translocation (for reviews, *see* {Mayer, 2005 #73; Schilke, 2006 #74; Vickery, 2007 #75}). Members of this protein family contain two domains, a N-terminal ATPase domain and a C-terminal peptide-binding domain. Central to all hsp70 functions is their ability to bind polypeptide substrates reversibly, and to use conformational changes driven by ATP binding and hydrolysis to regulate substrate affinity. ATP binding leads to a conformation that exhibits weaker substrate affinity and faster substrate exchange (*see* Figure 1.1), and subsequent hydrolysis to ADP and inorganic phosphate results in a conformational state with stronger substrate affinity and slower exchange {Hoff, 2000 #196; McCarty, 1995 #200; Palleros, 1993 #202; Schmid, 1994 #95; Silberg, 2001 #205; Takeda, 1996 #208}.

Mitochondria require hsp70 chaperones for the translocation of nuclear-encoded proteins {Matouschek, 2000 #123; Neupert, 2002 #124; Schneider, 1994 #125}, the synthesis of Fe-S-clusters {Dutkiewicz, 2006 #268; Muhlenhoff, 2003 #129; Muhlenhoff, 2002 #130; Schilke, 2006 #74}, and protein folding {Kang, 1990 #126}. In yeast, two chaperones (Ssc1 and Ssq1) contribute to these functions, whereas mammals have a

single hsp70 isoform (designated mtHsp70, HspA9b, and mortalin) that is predicted to fulfill these roles {Daugaard, 2007 #72; Kaul, 2007 #198}. Ssc1 and Ssq1 both appear to require the presence of a specialized hsp70 escort protein Hep1 (also designated Zim17 and Tim15) to maintain their solubility and perform their functions. *S. cerevisiae* containing an inactivated Hep1 exhibit a phenotype consistent with Ssc1 and Ssq1 depletion. This includes decreased import of nuclear-encoded proteins into the mitochondrial matrix, reduced activities of Fe-S proteins, and pleiotropic effects on mitochondrial morphology {Burri, 2004 #46; Sanjuan Szklarz, 2005 #29; Sichting, 2005 #47; Yamamoto, 2005 #48}. These phenotypes are thought to arise because yeast chaperones exhibit reduced solubility in the absence of Hep1.

Bacterial expression studies have provided evidence that eukaryotic escort proteins are sufficient to promote the solubility of their cognate hsp70 chaperones {Sichting, 2005 #47; Willmund, 2008 #44}. Coexpression of Ssc1 with Hep1 in *E. coli* led to the production of soluble Ssc1, whereas expression of Ssc1 alone yielded insoluble chaperone {Sichting, 2005 #47}. In addition, the *Chlamydomonas reinhardtii* chloroplast Hsp70B could only be produced as a soluble functional protein in bacteria when it was coexpressed with Hep2 {Willmund, 2008 #44}. Prior to this study, the nature of chaperone misfolding reactions and escort protein regulation of chaperone folding were unclear. It was unclear if escort protein activity arose from interactions with the Hsp70 peptide-binding domain, *i.e.*, with Hep1 serving as a substrate for the chaperone, or if escort activity could result from interactions with the Hsp70 ATPase domain. In addition, it was not clear if metazoan escort protein homologs can promote the solubility of their cognate chaperones similar to that observed in yeast and green algae, and it was not

known whether escort proteins elicit effects on chaperone and nucleotide interactions.

To better understand escort protein regulation of chaperone folding and function, I characterized the solubility of human mtHsp70, its isolated ATPase domain (designated 70^{ATPase}), and human-bacterial chaperone chimeras. In addition, I examined the effect of human Hep on the solubility of these proteins, and characterized the effect of Hep on mtHsp70 and nucleotide interactions.

2.2. Experimental procedures

2.2.1 Materials

E. coli XL1-Blue and Rosetta 2 cells were from Stratagene and EMD Biosciences, respectively. Enzymes for DNA manipulation were obtained from Roche Biochemical, New England Biolabs, and Promega. Synthetic oligonucleotides were obtained from Fischer Scientific, and pET vectors were from EMD Biosciences. NuPAGE Novex 10% Bis-Tris gels from Invitrogen were used for all electrophoresis experiments. Bacterial growth media components were from BD Biosciences, and all other reagents were from Sigma-Aldrich.

2.2.2. Vectors

The gene encoding human mtHsp70 was amplified from an Invitrogen Ultimate ORF Human Clone (accession #BC0004788) using VENT DNA polymerase and cloned into pET21d(+) using *ncoI* and *hindIII* restriction sites to generate pHsp70, a vector that produces full-length mtHsp70. The mtHsp70 gene and a gene encoding the mtHsp70 ATPase domain (residues 47 to 440) were also cloned into pET21d(+) using *NcoI* and

NotI to generate pHsp70-His and pATPase, respectively, vectors that produce mtHsp70 and its isolated ATPase domain with C-terminal His tags. All of these constructs produce mtHsp70 without its mitochondrial targeting sequence {Bhattacharyya, 1995 #132} but with an extra N-terminal methionine.

Gene fragments of human mtHsp70 and *E. coli* HscA were PCR amplified from pHsp70 and *E. coli* genomic DNA, splicing by overlap extension was used to generate chimeric chaperone genes {Horton, 1989 #197}, and these full-length chimeras were cloned into pET21d(+) using NcoI and NotI restriction enzymes. The first chimera, designated A-70 (pA-70), contained the HscA ATPase domain (residues 1-391) and the mtHsp70 peptide-binding domain (PBD; residues 441-679). The second chimera, designated 70-A (p70-A) contained the mtHsp70 ATPase domain (residues 47-440) and the HscA PBD (residues 392-616).

A vector (pHep-EGFP) that produces human Hep fused to EGFP was generated by chemically synthesizing the predicted human Hep gene (accession #NM_001080849) and cloning it into pEGFP-N1 using BglII and HindIII restriction endonucleases. In addition, the gene fragment encoding Hep without its predicted mitochondrial targeting sequence (residues 1 to 49) was PCR amplified and cloned into pET28b(+) and pET30a at *ncoI* and *hindIII* restriction sites. The pET28-derived vector (pHep) produces human Hep without an affinity tag, whereas the pET30-derived vector (pHis-Hep) produces human Hep with a N-terminal (His)₆ tag. This tag can be removed with enterokinase to produce the predicted mitochondrial isoform of Hep with an Ala-Met at its N-terminus. The *S. cerevisiae hep1* gene was amplified from genomic DNA using PCR and cloned into pET28b(+) using *ncoI* and *hindIII* restriction sites to create pHep1, a vector that produces

Hep1 (residues 48 to 174) without its mitochondrial targeting sequence {Sichting, 2005 #47}.

The gene encoding human mitochondrial Isu2 was PCR amplified from an Invitrogen plasmid (accession #BM921073) and cloned into pET30a using *ncolI* and *hindIII* restriction sites to create pHis-Isu2, a vector that produces Isu2 (residues 37 to 167) without its mitochondrial targeting sequence {Tong, 2000 #209} but with a N-terminal (His)₆ tag. All cloning and plasmid amplification was performed using a strain of *E. coli* (XL1-Blue; Stratagene Inc.) that lacks a T7 RNA polymerase and all constructs were sequence verified.

2.2.3 Solubility analysis

Protein solubility was analyzed using Rosetta 2 *E. coli* grown in LB. Cells transformed with vectors for expressing the indicated proteins were grown at 37°C, induced with 0.1 mM IPTG (isopropylthio-β-D-galactoside) at A₆₀₀ ≈ 1, and grown for ≈18 h at 23°C to allow expression. In experiments expressing yeast Hep1, cells were only allowed to grow for 6 hours after induction. Cells were harvested by centrifugation, resuspended in HED buffer (10 mM HEPES pH 7.5, 0.5 mM EDTA, and 1 mM DTT) containing 1 mM MgCl₂, 300 μg/mL lysozyme, 2 U/mL DNase I, and frozen at -80°C for ≥24 h. The lysed cells were thawed and centrifuged at 40k x g to separate the insoluble and soluble proteins. The insoluble fractions were resuspended in HED buffer, and protein concentrations in each fraction were determined using Bradford analysis prior to SDS-PAGE analysis.

2.2.4. Protein expression and purification

Cells transformed with pHis-Hep were grown at 37°C, induced with 0.1 mM IPTG at $A_{600} \approx 1$, and grown for ≈ 18 h at 23°C to allow for expression. Harvested cells were resuspended in TND buffer (50 mM Tris pH 8.0, 150 mM NaCl, and 1 mM DTT) containing 1 mM $MgCl_2$, 300 $\mu g/mL$ lysozyme, and 2 U/mL DNase I. After two freeze-thaw cycles at -80°C, lysed cells were centrifuged at 40k x g to remove cell debris. Cleared lysate was applied to Ni-NTA resin (Qiagen), the column was washed with TND buffer containing 15 mM imidazole, and (His)₆-tagged Hep1 was eluted using TND buffer containing 150 mM imidazole. After dialysis against 10 mM Tris pH 8.0, the fusion protein (15 mg/mL) was treated with 2 units/mL enterokinase (Promega) for 72 hours at room temperature to remove His-tag and applied to Histrap (GE Healthcare) column to remove affinity tag. Cleaved Hep was concentrated and chromatographed in TND buffer using a S75 Superdex column (GE Healthcare). Fractions appearing homogeneous were pooled and concentrated to >15mg/mL.

Rosetta 2 *E. coli* harboring pHsp70-His and pHep1 were grown and lysed using a similar protocol as described for Hep purification, except that TNED buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT) was used to resuspend cell pellets. After lysis, cells were centrifuged at 40k x g to remove cell debris, cleared lysate was applied to Ni-NTA resin, and the column was washed with 10 column volumes of TNED buffer containing 500 mM NaCl, 0.5% Triton X-100, and 10 mM imidazole. This latter step was required to remove yeast Hep1 that remained bound to mtHsp70 on the NTA column. Chaperone was eluted using TNED buffer containing 150 mM imidazole, fractions containing protein were combined, and ammonium sulfate was added to a final

concentration of 50% saturation to precipitate mtHsp70. The precipitated protein was resuspended in TED buffer (50mM Tris pH 8.0, 0.5 mM EDTA, and 1 mM DTT), centrifuged at 40k x g, dialyzed against TED buffer, and applied to a Q-sepharose column. Protein was eluted from this anion exchange column using a linear gradient from 0 to 400 mM NaCl in TED buffer. Fractions appearing homogenous were pooled, dialyzed against TED buffer, and concentrated to ~15 mg/mL. The mtHsp70 ATPase domain was expressed in Rosetta 2 *E. coli* harboring pATPase and pHep and purified using a procedure similar to that described for full-length mtHsp70.

2.2.5. Gel filtration chromatography

Protein molecular masses were estimated by comparing their elution to monomeric standards of known molecular weight on Superdex 75 and 200 columns, respectively, using an AKTA FPLC system (GE Healthcare). For experiments examining mtHsp70 oligomerization, 20 μ M chaperone was incubated for 30 minutes at 4°C in HKMD buffer (50 mM HEPES pH 7.5, 150 mM KCl, and 10 mM MgCl₂, and 1 mM DTT) containing and lacking 1 mM ADP or ATP. This sample was applied to a Superdex 200 column equilibrated in HKMD buffer and having levels of nucleotides (50 μ M) that allow for spectroscopic detection of the eluted proteins. In experiments examining Hep binding to mtHsp70, equimolar mtHsp70 (or its ATPase domain) and Hep were incubated in HKMD buffer for 30 minutes at 4°C to allow for complex formation. Nucleotides were then added to a final concentration of 1 mM and further incubated for 30 minutes prior to chromatographic separation in HKMD buffer containing 50 μ M ADP or ATP. For analysis in the absence of nucleotide, samples were immediately chromatographed in

HKMD buffer after the 30 minutes incubation. The standard curves shown were generated using the elution volumes for: amylase (200 kDa), alcohol dehydrogenase (158 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa).

2.2.6. Cell culture

HEK293 cells were cultivated in Dulbecco's Modification of Eagle's media (Fisher) supplemented with 10% bovine calf serum. The cells were incubated at 37°C in a 90% humidified atmosphere containing 5% CO₂. Before transfection (1 day), cells were seeded in 6-well plates and transfection was done at a cell confluency of 70% by using Fugene6 transfection reagent (Roche) according to the manufacturer's protocol. Mitochondria were stained 48 hours after transfection by incubating cells with 50 nM MitoTracker Red (Molecular Probes) in phosphate buffered saline PBS at 23°C for 30 min. After washing with PBS two times, live cells were imaged using LSM-510 (Zeiss) confocal fluorescence microscope. All images are nearly confocal (thin optical slice <1µm).

2.2.7 ATPase measurements

The rate of ATP hydrolysis was monitored as previously described using Invitrogen's EnzChek phosphate detection kit {Borges, 2003 #192; Oliveira, 2006 #201; Qiu, 2006 #203; Silberg, 2001 #205; Silberg, 1998 #270; Vickery, 1997 #190}. Assays were performed in HKMD buffer that contained 1 mM DTT. Enzymes and coupled assay reagents were incubated in a 0.5 mL reaction at 25°C for 5 min prior to starting the

reaction through addition of 1 mM ATP. First order rates were corrected for the degradation of the coupled enzyme substrate, 2-amino-6-mercapto-7-methylpurine ribonucleoside, as well as the level of phosphate present in ATP. Reaction rates obtained for mtHsp70 and its isolated ATPase domain were directly proportional to enzyme concentrations. Curves shown represent a least-squares fit of the data to a hyperbolic saturation function.

2.2.8. Analytical methods

Protein concentrations were determined spectrophotometrically. The extinction coefficients of mtHsp70 [$\epsilon_{280} = 19,600 \text{ M}^{-1}\text{cm}^{-1}$], the mtHsp70 ATPase domain [$\epsilon_{280} = 16,800 \text{ M}^{-1}\text{cm}^{-1}$], and Hep [$\epsilon_{280} = 9,800 \text{ M}^{-1}\text{cm}^{-1}$] were calculated using average absorptivities for tryptophan and tyrosine of 5,600 and 1,400 ($\text{M}\cdot\text{cm}$)⁻¹, respectively {Gill, 1989 #195; Mach, 1992 #199; Pace, 1995 #269}. All UV-Vis absorbance measurements were performed using a Cary 50 spectrophotometer.

2.3. Results

2.3.1. Domain requirements for mtHsp70 insolubility

Human mtHsp70 contains two domains, an N-terminal ATPase domain and a C-terminal peptide-binding domain, but the domain responsible for its low solubility upon expression in *E. coli* was not known. To investigate the relative contributions of these domains to mtHsp70 insolubility, I created bacterial expression vectors for mtHsp70 and chimeras of mtHsp70 and HscA, a constitutively expressed bacterial hsp70 family member that is soluble and monomeric {Vickery, 1997 #190}. The first chimera,

designated 70-A, contains a mtHsp70 ATPase domain and an HscA PBD (Figure 2.1). The second chimera, designated A-70, contains an HscA ATPase domain and a mtHsp70 PBD. In addition, I created an expression vector for a truncation mutant of mtHsp70 that contains only the ATPase domain.

Figure 2.2 shows the expression of mtHsp70, 70-A, A-70, and 70^{ATPase} in Rosetta 2 *E. coli* grown in LB medium. In all cases, IPTG-induced expression led to major bands with apparent molecular weights similar to those predicted. Analysis of the soluble and insoluble cell fractions indicated that mtHsp70 is predominantly within the insoluble fraction. In addition, the 70-A chimera and mtHsp70 ATPase domain were found within the insoluble fractions of the cells. The A-70 chimera, in contrast, was soluble under similar expression conditions. Taken together, these results suggest that the mtHsp70 ATPase domain contributes to the low solubility of full-length mtHsp70 under these assay conditions.

2.3.2. Hep-EGFP is localized to mitochondria

The N-terminal portion of Hep (residues 1-49) is predicted to constitute a mitochondrial localization signaling sequence {Claros, 1996 #193}, suggesting that this protein is translocated to mitochondria like the yeast escort protein Hep1 {Burri, 2004 #46}. To examine this, I created an expression vector that produced full-length Hep with enhanced green fluorescent protein (EGFP) fused to its C-terminus and examined the localization of Hep-EGFP in HEK293 cells transiently transfected with this vector. Figure 2.3 shows that the EGFP signal appears localized with that of MitoTracker Red, a dye that stains mitochondria by detecting the membrane potential.

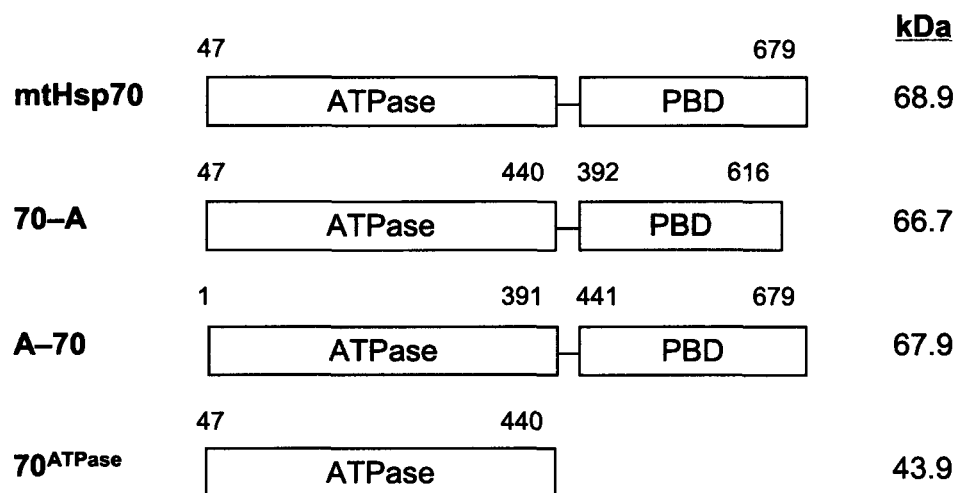


Figure 2.1. Chimeras and truncation constructed. The domain composition of chaperone constructs is shown with the residues derived from human mtHsp70 and *E. coli* HscA indicated. The calculated molecular weight for each protein is also shown.

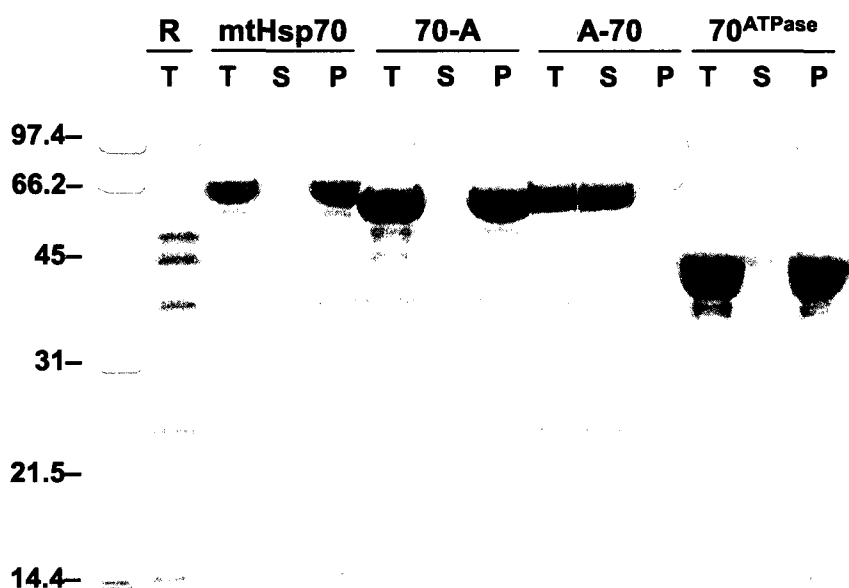


Figure 2.2. Solubility of mtHsp70, mtHsp70-HscA chimeras, and 70^{ATPase}. SDS-PAGE analysis of protein solubility upon expression in *E. coli*. Molecular weight standards (Lane 1), Rosetta 2 *E. coli* lacking plasmids (Lane 2), and Rosetta 2 *E. coli* harboring plasmids for expressing full-length mtHsp70 (pHsp70; Lanes 3-5), a chimera having an mtHsp70 ATPase domain and a HscA peptide-binding domain (p70-A; Lanes 6-8), a chimera having an HscA ATPase domain and a mtHsp70 peptide-binding domain (pA-70; Lanes 9-11), and the isolated mtHsp70 ATPase domain (pATPase; Lanes 12-14). For each sample, 15 μ g of total protein from whole cells (T) is shown as well as the soluble lysates (S) and pellets (P) derived from a sample that contained 15 μ g of protein before fractionation.

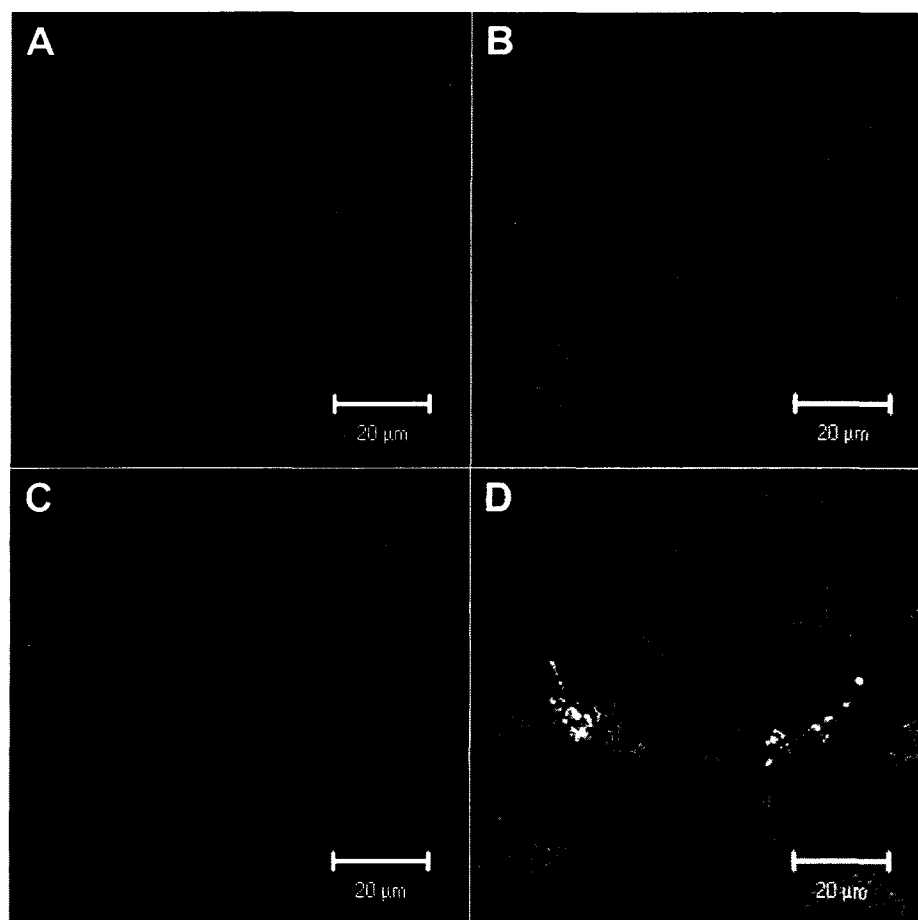


Figure 2.3. Mitochondrial localization of human Hep. HEK293 cells transiently transfected with pHep-EGFP were stained by 50 nM mitotracker RED for 30 minutes at 48 hours after transfection. Live cells were imaged using a confocal microscope in the presence of phosphate buffered saline. *A*, EGFP channel representing Hep-EGFP fusion protein. *B*, bright field, *C*, mitotraker RED channel representing mitochondria, and *D*, overlap of *A*, *B* and *C*. The mitotracker dye was excited with a 543 nm argon laser with emission collected through a 560 nm long-pass filter, and the EGFP was imaged using 488 excitation and emission detected through a 500 to 530 nm band pass. The scale bar represents 20 μm .

2.3.3. Hep enhances mtHsp70 solubility

Expression studies with yeast Ssc1 have shown that this chaperone can only be produced as a soluble recombinant protein in *E. coli* when it is coexpressed with yeast Hep1 {Sichting, 2005 #47}, implicating escort proteins as sufficient to maintain mitochondrial chaperone solubility. To see if human Hep exhibits hsp70 escort activity, I created a bacterial expression vector that produces the predicted mitochondrial isoform of Hep with a cleavable N-terminal His tag {Claros, 1996 #193}, and I evaluated the escort activity of this recombinant protein using a bacterial expression assay.

Figure 2.4 shows an SDS-PAGE of total protein from cells overexpressing Hep. A major band is observed that migrates with an apparent molecular weight that is heavier than that calculated for His-tagged Hep (18.6 kDa), indicating that Hep migrates slower than expected under these conditions. Previous studies analyzing yeast Hep1 migration on SDS-PAGE have found similar results {Sichting, 2005 #47}, suggesting that abnormalities in SDS binding or protein conformation during electrophoresis are responsible for the slower than expected migration of these escort proteins. In addition, Hep is predominantly within the soluble fraction when expressed alone. Figure 2.4 also shows the effect of Hep on the solubility of mtHsp70, 70-A, and 70^{ATPase}. In all three cases, more than half of the expressed chaperone was observed in the soluble cellular fraction. This can be contrasted with experiments performed in the absence of Hep, where I could not detect soluble mtHsp70, 70-A, or 70^{ATPase} (see Figure 2.2). These findings provide evidence that human Hep exhibits chaperone escort activity similar to that observed with yeast Hep1 {Sichting, 2005 #47}. Furthermore, they suggest that Hep regulates chaperone solubility through interactions with the mtHsp70 ATPase domain.

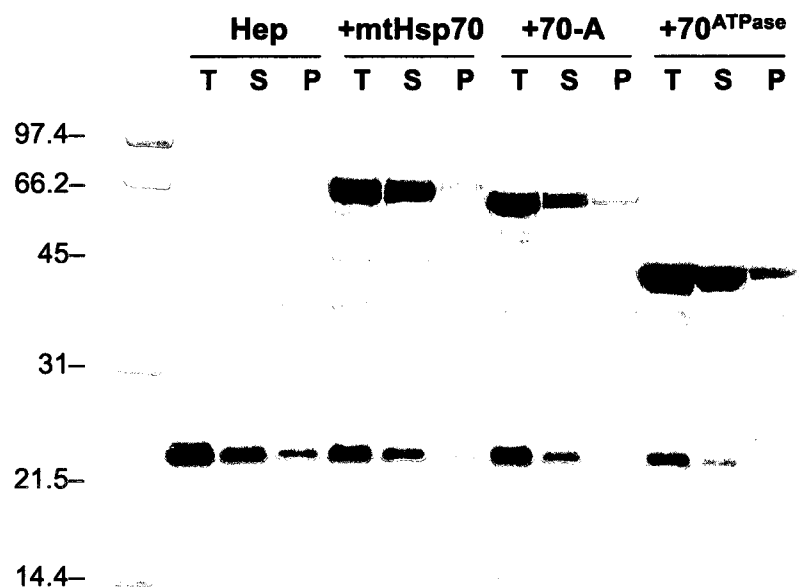


Figure 2.4. SDS-PAGE analysis of human Hep escort activity. Molecular weight standards (Lane 1) and Rosetta 2 *E. coli* harboring a plasmid for expressing Hep (pHis-Hep; Lanes 2-4) and vectors for expressing mtHsp70 (pHsp70; Lanes 5-7), the 70-A chimera (p70-A; Lanes 8-10), and the ATPase domain from mtHsp70 (pATPase; Lanes 11-13). For each sample, 15 μ g of total protein from whole cells (T) is shown as well as the soluble lysates (S) and pellets (P) derived from a sample that contained the same amount of protein before fractionation.

Figure 2.5 shows the effect of a non-cognate escort protein (*S. cerevisiae* Hep1) and a substrate (human Isu2) on the solubility of human mtHsp70 expressed in *E. coli*. IPTG-induced expression of both Hep1 and Isu2 led to major bands with apparent molecular weights slightly larger than those predicted for Isu2 ($M_r = 19.2$ kDa) and Hep1 ($M_r = 14.6$ kDa), as previously observed with yeast Hep1 {Sichting, 2005 #47} and a bacterial homolog of Isu2 {Hoff, 2000 #196}. In addition, both proteins were predominantly in the soluble cell fractions. Although both proteins were expressed at similar levels, only the yeast escort protein Hep1 was able to promote human mtHsp70 to a similar extent as human Hep. In contrast, coexpression of mtHsp70 with Isu2 had little influence on chaperone solubility.

The slow migrations of Hep1 and Isu2 during electrophoresis are thought to arise from abnormalities in SDS binding or protein conformation, not retention of bound cofactors. Hep1 mutants having zinc-chelating cysteines mutated to serine exhibit similar migration during electrophoresis as native Hep1, even though these mutations abolish the solubility of recombinant Hep1. In addition, the Fe-S clusters coordinated by Isu2-type proteins are unstable in the presence of the oxygen levels where gel electrophoresis was performed {Hoff, 2000 #196}.

2.3.4. Protein expression and purification

To obtain soluble mtHsp70 for purification, I coexpressed this chaperone with yeast Hep1 in *E. coli*. This approach was used because Hep1 promotes mtHsp70 solubility like human Hep, but it does not remain as strongly bound to the chaperone during chromatography, aiding in the separation of the escort protein from the chaperone during

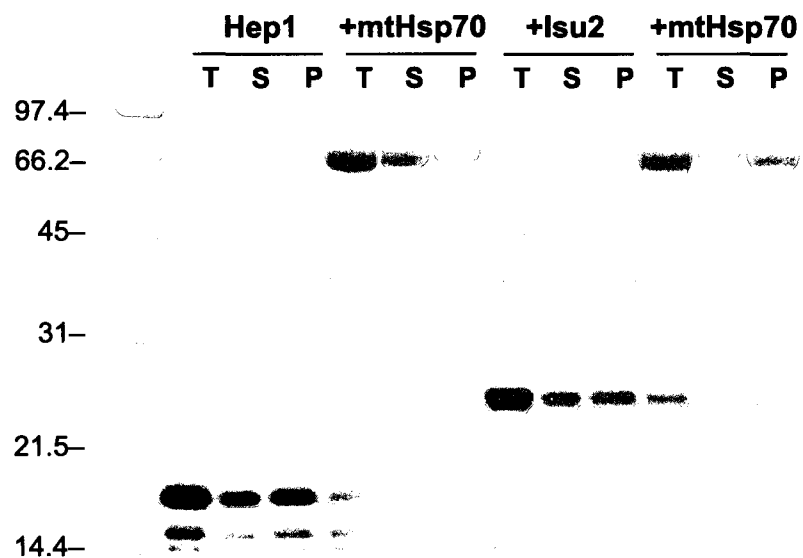


Figure 2.5. SDS-PAGE analysis of yeast Hep1 and human Isu2 escort activity. Molecular weight standards (Lane 1) and Rosetta 2 *E. coli* harboring plasmids for expressing yeast Hep1 (pHep1; Lanes 2-4), yeast Hep1 and human mtHsp70 (pHep1 and pHsp70; Lanes 5-7), human Isu2 (pHis-Isu2; Lanes 8-10), and human Isu2 and mtHsp70 (pHis-Isu2 and pHsp70; Lanes 11-13). For each sample, 15 μ g of total protein from whole cells (T) is shown as well as the soluble lysates (S) and pellets (P) derived from a sample that contained the same amount of protein before fractionation.

purification. In addition, the mtHsp70 ATPase domain was expressed in the presence of human Hep lacking a His tag. Hep lacking this affinity tag promotes 70^{ATPase} and mtHsp70 solubility upon expression in *E. coli*, but it is expressed at lower levels than His-tagged Hep. This lower expression aids in chromatographic separation of 70^{ATPase} and Hep during purification.

Figures 2.6A, 2.7A, and 2.8A show SDS-PAGE analysis of the final preparations of purified mtHsp70, Hep, and 70^{ATPase}, respectively. In all cases, a single major band is obtained indicative of a high level of purity. In addition, I characterized the absorption spectrum of each protein. They exhibited spectra consistent with the known amino acid content of aromatic residues and showed no evidence for high levels of additional chromophoric groups, *e.g.*, bound ADP/ATP that contributes significantly to absorption at 260 nm, as has been observed in purified preparations of other hsp70 chaperones like *E. coli* DnaK {Russell, 1998 #204}.

Subsequent to purification, mtHsp70 and 70^{ATPase} remained soluble in the absence of Hep and nucleotides when stored in Tris pH 8. In the case of the ATPase domain, the protein remained soluble at concentrations as high as 200 μ M even when stored for 24 hours at room temperature. However, the mtHsp70 ATPase domain could not be stably stored at high concentrations for a similar period of time in buffers having pH values (\approx 7.5) more closely resembling the physiological environment within *E. coli* where these proteins were overexpressed, unless it was incubated with equimolar Hep.

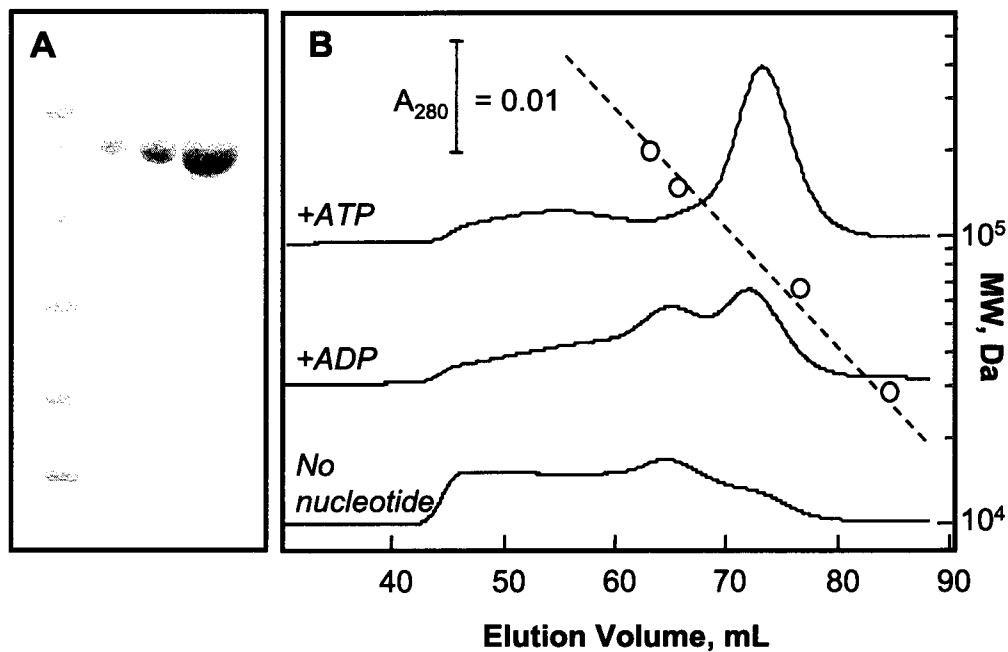


Figure 2.6. Nucleotide effects on mtHsp70 oligomerization. *A*, SDS-PAGE analysis of purified mtHsp70: molecular weight standards (Lane 1) and 1, 3, and 10 µg of purified mtHsp70 (Lanes 2-4). *B*, Elution profiles at 4 °C for mtHsp70 (20 µM in 2 mL) chromatographed on a Superdex 200 column using HKMD buffer containing 50 µM ATP (top), 50 µM ADP (middle), and no nucleotide (bottom).

2.3.5. Gel filtration analysis

Hsp70 binding to ATP results in a conformation change that leads to the formation of a tense state with reduced affinity for peptide substrates, and subsequent ATP hydrolysis to ADP results in formation of a relaxed state with increased substrate affinity {Hoff, 2000 #196; McCarty, 1995 #200; Palleros, 1993 #202; Schmid, 1994 #95; Silberg, 2001 #205; Takeda, 1996 #208}. For some chaperones, like yeast mitochondrial Ssc1, these nucleotide-induced conformational changes influence the oligomeric state of the chaperone {Sichting, 2005 #47}, suggesting that human mtHsp70 may also self-associate in a nucleotide-dependent manner. To evaluate this possibility, I investigated mtHsp70 migration on a gel filtration column in the presence and absence of ATP and ADP.

Figure 2.6B shows elution profiles for mtHsp70 on a Superdex 200 size-exchange column. In the presence of 50 μ M ATP, a single major peak was observed. A comparison of this peak's elution volume to protein standards of known size indicates that mtHsp70 exhibits an apparent molecular mass (81 kDa) that is similar to the calculated mass for a mtHsp70 monomer. In the presence of ADP, two major peaks were observed at elution volumes with predicted molecular masses (86 and 168 kDa), consistent with the presence of mtHsp70 monomers and dimers. Nucleotide-free mtHsp70 also contained a mixture of monomeric and dimeric mtHsp70. Under these conditions, however, a larger fraction of the mtHsp70 eluted near the void volume (43.9 mL) of this column. This indicates that mtHsp70 forms higher order oligomers in the absence of nucleotides.

To evaluate whether Hep influences mtHsp70 oligomerization, I investigated whether Hep altered mtHsp70 elution on a Superdex 200 size-exclusion column. Figure 2.7B compares the elution of Hep and a mixture of Hep and mtHsp70. In the absence of

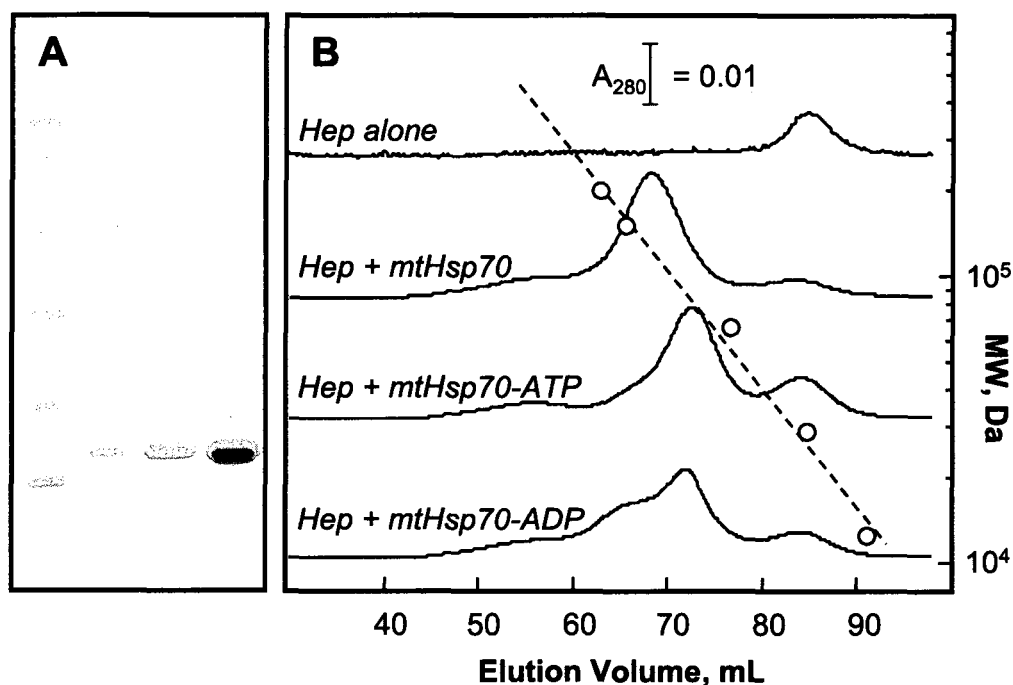


Figure 2.7. Hep inhibits mtHsp70 oligomerization. *A*, SDS-PAGE analysis of purified mtHsp70: molecular weight standards (Lane 1) and 1, 3, and 10 μ g of purified Hep (Lanes 2-4). *B*, Effect of mixing Hep and mtHsp70 on their elution at 4 $^{\circ}$ C from a Superdex 200 column chromatographed using HKMD buffer lacking nucleotides and containing ATP or ADP (50 μ M). Samples introduced onto the column (2 mL) contained equimolar concentrations (20 μ M) of Hep and mtHsp70.

nucleotide, the protein mixture eluted as a single major peak. This can be contrasted with elution of mtHsp70 alone, which eluted as a mixture of monomers, dimers, and higher-order oligomers (*see* Figure 2.6B). A comparison of the elution volume for this peak to that of the standards indicates that the Hep-mtHsp70 complex exhibits an apparent molecular mass (124 kDa) that is less than that expected for a mtHsp70 dimer, suggesting that Hep promotes formation of mtHsp70 monomers. Hep alone eluted as a monodisperse peak at an apparent molecular mass (29 kDa) that is ~2-fold greater than that calculated for a Hep monomer after removal of its His-tag (13.6 kDa).

To determine whether Hep forms a stable complex with the mtHsp70 ATPase domain, I examined whether Hep altered 70^{ATPase} elution from a Superdex 75 size-exclusion column. Figure 2.8B shows a comparison of the peaks obtained to protein standards. When chromatographed alone, 70^{ATPase} displayed an apparent molecular weight (~54 kDa) similar to that calculated for a monomer (43.9 kDa). This indicates that this domain does not self-associate like the full-length protein (*see* Figure 2.6B). In addition, an equimolar mixture of 70^{ATPase} and Hep eluted at a single volume greater than that observed for 70^{ATPase} or Hep alone. Under these conditions, little free Hep or 70^{ATPase} was detected. This suggests that these proteins bind with a stoichiometry of 1:1, since non-stoichiometric binding for an equimolar mixture would have yielded two major peaks, one representing the 70^{ATPase} that forms a complex with Hep and the other representing the remaining free 70^{ATPase}. The finding that Hep migrates with an apparent molecular weight similar to that of a dimer further suggests that Hep must dissociate prior to binding to 70^{ATPase}, or that Hep has an asymmetrical shape that causes it to migrate more rapidly during gel filtration chromatography than a symmetrical monomer.

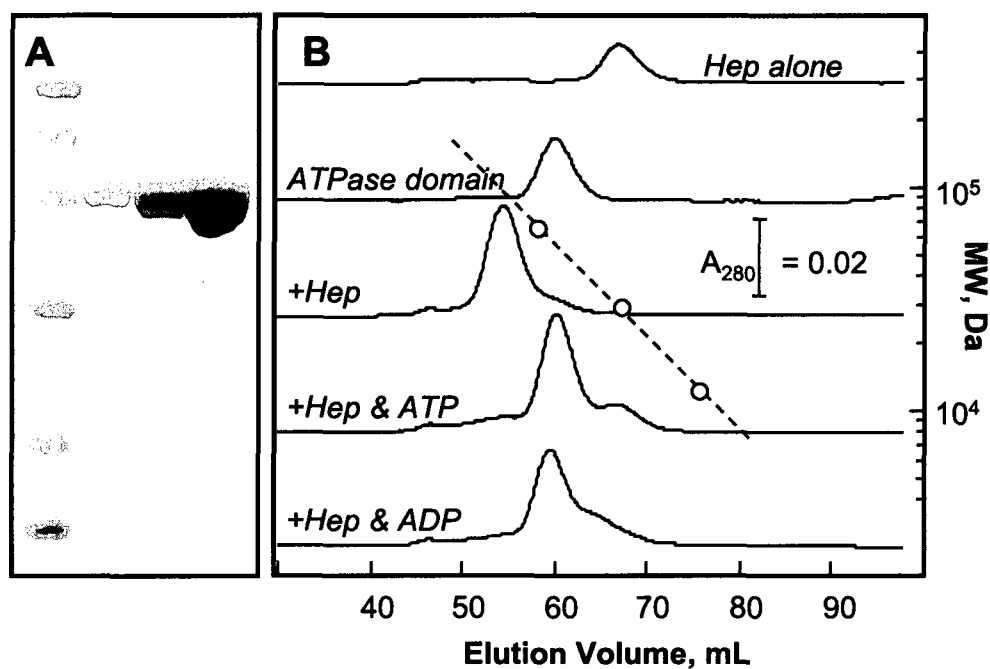


Figure 2.8. Hep binds to the mtHsp70 ATPase domain. *A*, SDS-PAGE analysis of purified mtHsp70 ATPase domain: molecular weight standards (Lane 1) and 1, 3, and 10 μg of purified ATPase domain (Lanes 2-4). *B*, Elution profiles at 4 $^{\circ}\text{C}$ for the mtHsp70 ATPase domain and Hep chromatographed alone and together on a Superdex 75 column using HKMD buffer lacking nucleotides and containing ATP or ADP (50 μM). Each sample chromatographed (2 mL) introduced onto the column contained 20 μM Hep and 70^{ATPase}.

2.3.6. Nucleotide effects on Hep binding

The finding that Hep binds directly to the mtHsp70 ATPase domain suggested that nucleotide-induced conformational changes could influence the stability of the Hep-mtHsp70 complex. To address this, gel filtration analysis of the escort-chaperone complexes were repeated in buffers containing ADP and ATP. Figure 2.7B shows that the mtHsp70-Hep mixture eluted as two peaks in the presence of ATP. This can be contrasted with experiments performed in buffer lacking nucleotides, where mtHsp70 and Hep eluted together as a single major peak. In the presence of ATP, the faster peak eluted at a volume identical to that observed for mtHsp70-ATP chromatographed alone (*see* Figure 2.6B), and the slower peak eluted at a volume identical to that observed when Hep was analyzed alone. In the presence of ADP, multiple peaks were also observed. Again, elution of these peaks occurred at volumes similar to those observed in experiments analyzing the migration of the individual proteins.

I also investigate whether nucleotides promoted dissociation of Hep from the mtHsp70 ATPase domain. Figure 2.8B shows the effects of ADP and ATP on the elution of Hep and 70^{ATPase} mixtures from a size-exclusion column. In an ATP containing buffer, the Hep-70^{ATPase} mixture eluted as two distinct peaks at volumes corresponding to the elution observed when experiments were performed with the individual proteins. This can be contrasted with experiments performed in the absence of nucleotides where the Hep-70^{ATPase} complex eluted as a single peak at a smaller volume. In addition, gel filtration analysis in a buffer containing ADP resulted in multiple elution peaks. However, the two peaks observed under these conditions were not as resolved as in the presence of ATP, suggesting that ADP-bound 70^{ATPase} binds Hep stronger than ATP-bound protein.

2.3.7. ATPase activity

To investigate whether Hep affects mtHsp70 and nucleotide interactions, I examined the effect of Hep on the ATPase activity of mtHsp70 and its isolated ATPase domain using a coupled enzyme assay for phosphate release {Silberg, 2001 #205; Silberg, 1998 #270; Vickery, 1997 #190}. Figure 2.9 shows the effect of 40 μ M Hep on the rate of ATP hydrolysis catalyzed by 70^{ATPase} (2 μ M) in the presence of 1 mM ATP. Under these conditions, Hep increased the activity of 70^{ATPase} ~11-fold from 0.0054 to 0.062 mol ATP hydrolyzed per mol 70^{ATPase} per second. Purified Hep alone exhibited a low background level of ATPase activity under these conditions ($6 \times 10^{-5} \text{ s}^{-1}$).

To determine the affinity of the mtHsp70 ATPase domain for Hep, ATPase measurements were carried out over a range of concentrations of Hep. Figure 2.10 shows the increase in ATPase activity relative to the rate in the absence of Hep as a function of Hep concentration. Assuming a 1:1 stoichiometry for the binding of Hep to 70^{ATPase}, as predicted from size-exclusion chromatography measurements and observed during calorimetric analysis of Hep and 70^{ATPase} binding (*see* Figure 2.11), a hyperbolic saturation curve was obtained when the data was corrected for the amount of Hep bound. Extrapolation to saturating levels of Hep indicates a maximal stimulation of 11.5-fold with half-maximal stimulation at 7.6 μ M Hep.

Figure 2.12 shows the ATPase activity of full-length mtHsp70 (10 μ M) in the absence and presence of 40 μ M Hep. The basal activity of mtHsp70 ($6.8 \pm 0.2 \times 10^{-4}$ mol ATP hydrolyzed per mol per mtHsp70 per second) was less than that of the isolated ATPase domain, as observed with other hsp70 family members {Silberg, 2001 #205}. In addition, Hep (40 μ M) elicited a greater stimulation (~20-fold) of full-length mtHsp70 under these

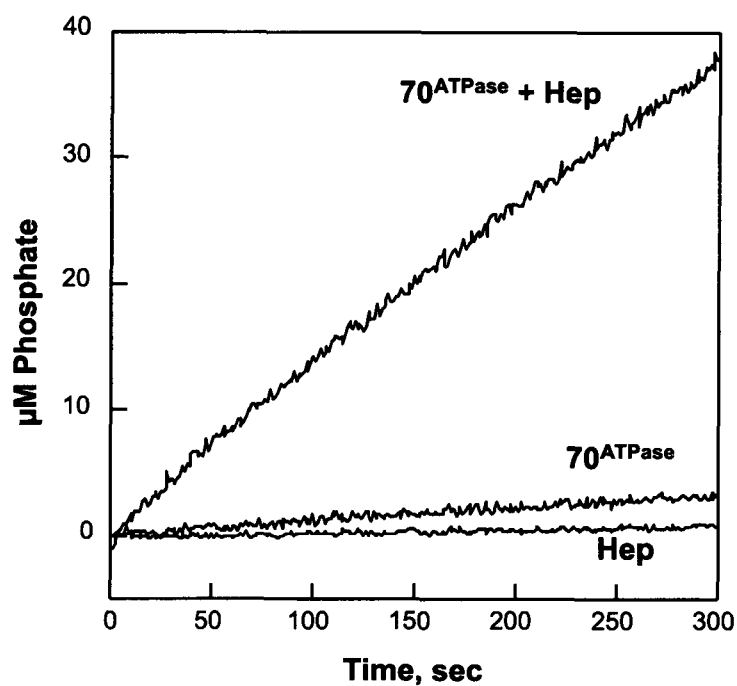


Figure 2.9. Hep stimulates the ATPase activity of 70^{ATPase}. A time course of ATP hydrolysis at 25 °C in reactions containing 2 μM 70ATPase, a mixture of 2 μM 70^{ATPase} and 40 μM Hep, and 50 μM Hep. Rates were measured in HKMD buffer that contained 1 mM ATP.

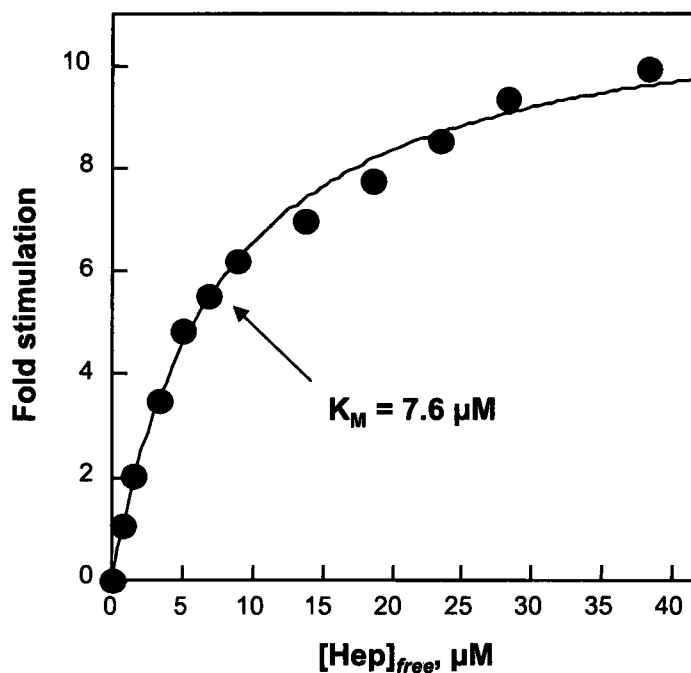


Figure 2.10. Effect of Hep concentration on the ATPase activity of 70^{ATPase}. The increase in the basal ATPase rate of 70^{ATPase} (2 μM) at 25 °C in the presence of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, and 40 μM Hep. All experiments were performed in HKMD buffer that contained 1 mM ATP. The concentration of free Hep was calculated assuming 1:1 binding stoichiometry with 70^{ATPase} using the equation: $[\text{Hep}]_{\text{free}} = [\text{Hep}]_{\text{total}} - \Delta v[E]/\Delta v_{\text{max}}$ where $[\text{Hep}]_{\text{total}}$ is the concentration of Hep added to the reaction, $[E]$ is the concentration of 70^{ATPase}, Δv is the observed change in rate, and Δv_{max} is the maximal rate obtained by extrapolating to infinite Hep concentration. The data are fit to a hyperbolic saturation function assuming a maximal stimulation for 70^{ATPase} of 11.5 fold with half maximal stimulation at 7.6 μM. Hep (50 μM) yielded a turnover number (0.00006 s⁻¹) that was ~100-fold lower than the basal rate measured for 70^{ATPase} (0.0054 s⁻¹).

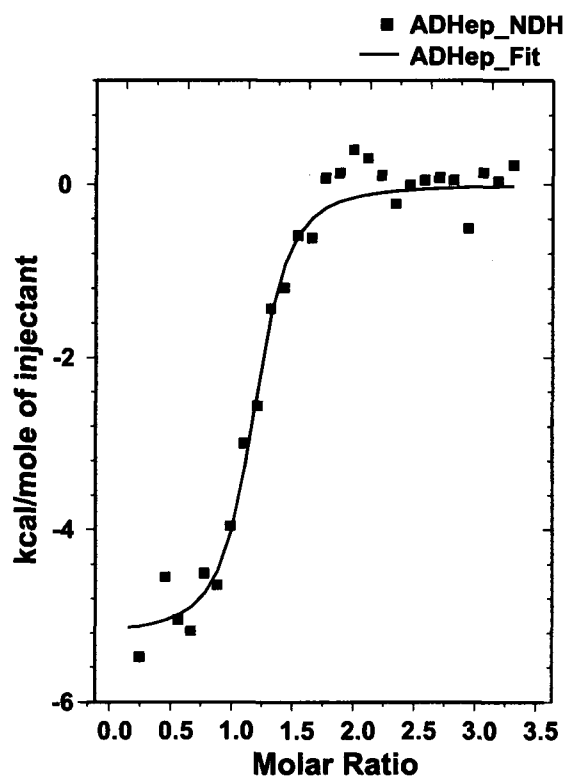


Figure 2.11. Calorimetric analysis of Hep and mtHsp70 binding. Heat generated upon successive additions of Hep to a solution containing the mtHsp70 ATPase domain. The data are plotted as the integrated heats of binding (corrected for the heat of dilution) versus the molar ratio of Hep to 70^{ATPase}. The solid line was generated as previously described (Silberg et al, 2001) and corresponds to binding of 1.02 mol Hep per mol of 70^{ATPase} with a $K_D = 135$ nM.

conditions. Furthermore, biphasic ATP hydrolysis kinetics was observed when reactions were started by adding ATP to mixtures of mtHsp70 and Hep that had been preincubated for 5 minutes. This can be contrasted with ATP hydrolysis by 70^{ATPase} in the presence of Hep, which was linear over the time course of the assay. Reactions involving full-length mtHsp70 yielded linear rates when mtHsp70 was incubated with ATP prior to Hep addition.

To examine whether mtHsp70 exhibits a similar affinity for Hep as 70^{ATPase} , I examined the effect of Hep concentration on mtHsp70 ATPase activity. Figure 2.13A shows the increase in the rate of ATP hydrolysis during the first 30 seconds of the reactions relative to the rate in the absence of Hep as a function of Hep concentration. In all reactions containing Hep, the fast phase exhibited linear rates over this time course. Extrapolation to saturating levels of Hep indicates a maximal stimulation of mtHsp70 activity (~49-fold) that is greater than that observed with the ATPase domain alone. However, the concentration of Hep required for half-maximal stimulation (5 μM Hep) is similar to that observed with 70^{ATPase} . Figure 2.13B shows the effect of Hep on mtHsp70 activity after the reaction was allowed to proceed for two minutes. During this time, Hep stimulated mtHsp70 activity to a lower maximal extent (27.6-fold), and the concentration of Hep required for half-maximal stimulation was ~5-fold greater (~26 μM) than that observed when analyzing the initial rates. In all reactions, ATP hydrolysis rates were linear between two and five minutes.

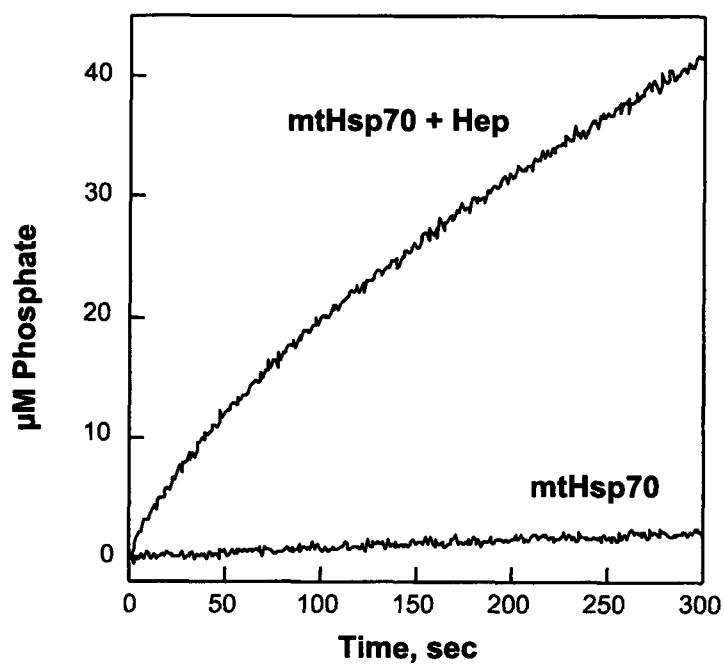


Figure 2.12. Hep stimulates full-length mtHsp70 ATPase activity. A time course of ATP hydrolysis at 25 °C in reactions containing 10 μM mtHsp70 and a mixture of 10 μM mtHsp70 and 40 μM Hep. Rates were measured at 25 °C in HKMD buffer containing 1 mM ATP.

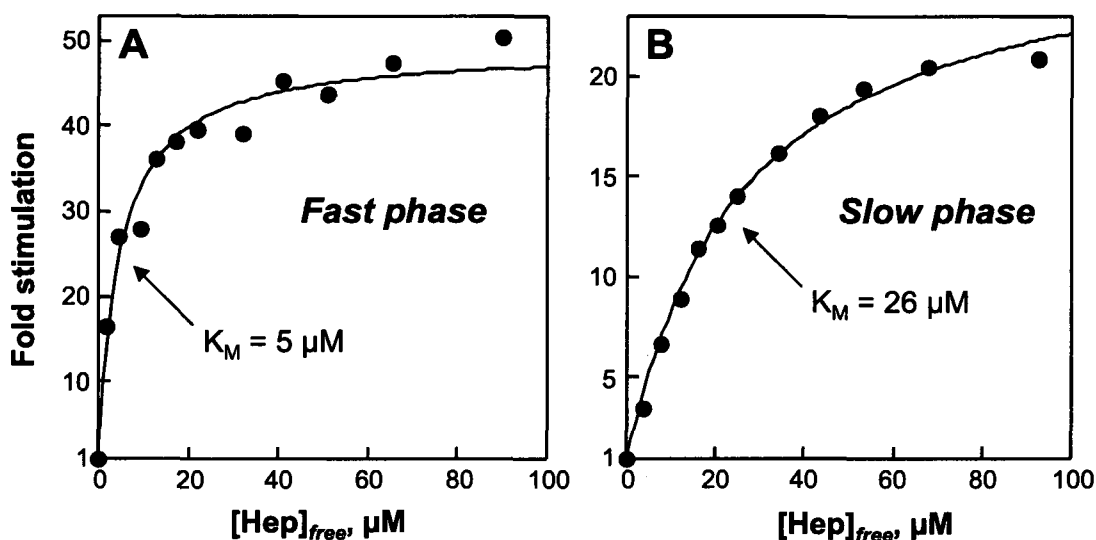


Figure 2.13. Effect of Hep concentration on the ATPase activity of mtHsp70. *A*, the effect of increasing Hep on the ATPase rate of mtHsp70 (10 μM) observed during the fast kinetic phase (0 to 30 seconds). *B*, the effect of increasing Hep on the ATPase rate of mtHsp70 (10 μM) observed after reactions had proceeded for 150 seconds. Reactions were performed at 25 °C in HKMD buffer containing 1 mM ATP, 10 μM mtHsp70, and 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, and 100 μM Hep. The concentration of free Hep was calculated assuming a 1:1 binding stoichiometry as described in Figure 8, and the data in *A* and *B* were fit to a hyperbolic saturation functions assuming a maximal stimulation for the faster kinetic phase of 49-fold with half maximal stimulation at 5 μM, and a maximal stimulation for the slower kinetic phase of 27.6-fold with half maximal stimulation at 26 μM.

2.4. Discussion

In earlier studies, escort proteins from yeast mitochondria (Hep1) and algae chloroplasts (Hep2) were shown to maintain their cognate chaperones in an active conformation by preventing chaperone aggregation {Burri, 2004 #46; Sanjuan Szklarz, 2005 #29; Willmund, 2008 #44; Yamamoto, 2005 #48}. However, the domain(s) responsible for aggregation and escort protein binding were not established. Our results herein provide evidence that the N-terminal ATPase domain of human mtHsp70 is responsible for its reduced solubility. Like full-length mtHsp70, a truncation mutant containing only the ATPase domain was insoluble when overexpressed in *E. coli*, as was the 70-A chimera containing the human mtHsp70 ATPase domain fused to the C-terminal peptide-binding domain of *E. coli* HscA, a monomeric hsp70-family member that has evolved to regulate Fe-S-cluster biosynthesis reactions {Vickery, 2007 #75; Vickery, 1997 #190}. In contrast, a chimera having the mtHsp70 PBD fused to an HscA ATPase domain was completely soluble upon overexpression in *E. coli*. These findings are consistent with studies performed by Craig and coworkers, which showed that an mtHsp70 truncation mutant having only the C-terminal PBD can be readily expressed as a soluble protein in the absence of Hep {Schilke, 2006 #74}.

Our findings also provide the first direct evidence that escort proteins promote chaperone solubility through interactions with the ATPase domain. Hep enhanced the solubility of full-length mtHsp70, a truncation mutant containing only the ATPase domain, and the 70-A chimera that contained a human mtHsp70 ATPase domain. In contrast, mtHsp70 solubility was not dramatically altered by coexpression with Isu2. In previous studies, Isu2-type proteins have been shown to serve as substrates for

mitochondrial hsp70 family members {Cupp-Vickery, 2004 #194; Dutkiewicz, 2003 #168; Silberg, 2001 #205}. In addition, a recent study showed that the C-terminal peptide-binding domain of mtHsp70 binds to a polypeptide harboring a LPPVK motif, the minimal portion of Isu2-type proteins that is required for chaperone binding {Hoff, 2002 #191; Schilke, 2006 #74}. The low chaperone escort activity elicited by Isu2 suggests that efficient suppression of mtHsp70 aggregation requires a protein that interacts with its N-terminal ATPase domain.

To our surprise, I found that *S. cerevisiae* Hep1 could promote the solubility of human mtHsp70. The ability of yeast Hep1 to cross react with human mtHsp70 suggests that mammalian and yeast escort protein homologs have similar topology {Momose, 2007 #45}, even though they display only 25% amino acid sequence identity. This also suggests that these escort protein homologs use a similar mechanism to bind their cognate chaperones, and it implicates a role for residues conserved among these homologs in mediating chaperone interactions. Support for this comes from a recent study evaluating the solubility and escort activity of yeast Hep1 mutants having residues conserved in the human homolog mutated, including H107 and D111 {Momose, 2007 #45}. Yeast Hep1 variants harboring these mutations all exhibited parent-like solubility when produced in yeast and *E. coli*, suggesting that these residues are not required for proper folding. In addition, Hep1 variants containing the D111A mutation (or a H107A mutation in combination with R106A) were impaired in their ability to promote the solubility of Ssc1 overexpressed in *E. coli*, and they could not support the growth of $\Delta hep1$ *S. cerevisiae* {Momose, 2007 #45}.

Gel filtration studies evaluating chaperone and escort protein binding revealed that

mtHsp70-Hep and 70^{ATPase}-Hep complexes are most stable in the absence of nucleotides. These measurements also revealed that Hep inhibits the self-association of mtHsp70 in the absence of nucleotides. This self-association is thought to arise at least in part from mtHsp70-mtHsp70 interactions involving the ATPase and substrate-binding domains of different chaperone molecules, since 70^{ATPase} did not self-associate in buffers lacking nucleotides. The former mirrors findings from a study examining the effect of nucleotides and Hep on the oligomerization of the yeast mitochondrial chaperone Ssc1 {Sichting, 2005 #47}. In the absence of nucleotide, glutaraldehyde crosslinking treatment generated Ssc1 that migrated on SDS-PAGE with a molecular weight consistent with the presence of 4 or more proteins per complex. However, in the presence of Hep1 (or ADP and ATP), a majority of the Ssc1 was monomeric or dimeric, indicating that Hep1, ADP, and ATP inhibit the formation of higher order Ssc1 oligomers.

Purified mtHsp70 exhibited slow intrinsic ATPase activity ($6.8 \pm 0.2 \times 10^{-4} \text{ s}^{-1}$) characteristic of hsp70 family members {Silberg, 2000 #207}, and this activity was enhanced by Hep. This implicates a role for Hep-type escort proteins in regulating chaperone and nucleotide interactions. This stimulation is predicted to arise at least in part from Hep binding to the ATPase domain of mtHsp70, since Hep was capable of stimulating the ATP hydrolysis activity of the isolated ATPase domain (up to 11.5-fold) and full-length mtHsp70 (up to 49-fold). Hep activation of mtHsp70 activity could arise from Hep stimulating the rate of ATP hydrolysis, similar to that reported for J-type auxiliary cochaperones {Misselwitz, 1998 #93; Silberg, 2000 #207}. Alternatively, Hep could enhance the rate of nucleotide exchange, similar to GrpE-type cochaperones {Liberek, 1991 #105}. Both J and GrpE-type family members are present in the

mammalian mitochondria {Borges, 2003 #192; Oliveira, 2006 #201; Qiu, 2006 #203}, suggesting that Hep may cooperate or compete with these proteins in regulating mtHsp70 ATPase activity.

Full-length mtHsp70 exhibited linear ATP hydrolysis kinetics in the absence of Hep. In contrast, biphasic kinetics was observed in reactions involving Hep and mtHsp70. These two linear kinetic phases were stimulated to different extents by Hep. In the case of the fast phase, the rate of ATP hydrolysis was enhanced up to 49-fold with half-maximal stimulation occurring at 5 μ M Hep. In contrast, the rate of ATP hydrolysis during the slower phase was only stimulated up to 27.6-fold with a higher concentration (26 μ M) of Hep required for half-maximal stimulation. The stronger apparent affinity for the fast kinetic phase suggests that mtHsp70 and Hep form a complex in the absence of nucleotides that has a distinct conformational state from ATP-bound mtHsp70. In addition, the higher stimulation observed for the fast kinetic phase suggests that Hep directly stimulates the rate of ATP hydrolysis ~49-fold during the first round of substrate turnover, and it implicates a subsequent ATPase reaction cycle step as rate-limiting during subsequent rounds of ATP hydrolysis. Additional studies are needed to establish the exact role that Hep plays in regulating the individual steps of the mtHsp70 ATPase reaction cycle.

Chapter 3

A conserved histidine in human Hep is required for stimulation of mitochondrial Hsp70 ATPase activity

Abstract

The hsp70 escort protein Hep increases the solubility and ATPase activity of the mitochondrial molecular chaperone mtHsp70. To identify Hep residues that are critical for chaperone regulation, I carried out an alanine mutagenesis scan of eight charged residues in a tryptophan-free mutant (W115I) of human Hep and assessed the effect of each mutation on Hep interactions with mtHsp70. Binding studies examining the effect of escort protein mutants on chaperone tryptophan fluorescence identified three mutations (R81A, H107A, and D111A) that decrease the binding affinity of Hep for nucleotide-free chaperone. In addition, steady-state ATPase measurements revealed that the H107A mutant fails to elicit an increase in the steady-state activity of mtHsp70. However, all mutations retain *in vitro* escort activity. I also examined whether Hep mutants with impaired binding to mtHsp70 can function in mitochondria by characterizing their ability to support the growth of $\Delta hep1$ *S. cerevisiae*. Complementation studies revealed that Hep-H107A does not support the growth of $\Delta hep1$ yeast like human Hep and the R81A and K88A mutants. These findings provide evidence that human Hep is a functional ortholog of yeast Hep1, and they implicate H107 as critical for human Hep regulation of mitochondrial hsp70 chaperones.

3.1. Introduction

In mammals, decreased expression of the mitochondrial hsp70 molecular chaperone (mtHsp70) leads to cellular senescence (Wadhwa et al., 2004) and has been correlated with the progression of Parkinson disease (Jin et al., 2006; Shi et al., 2008), while increased expression has been associated with greater malignancy of cancers (Czarnecka et al., 2006; Wadhwa et al., 2006; Yi et al., 2008) and extended lifespans (Kaul et al., 2003; Yokoyama et al., 2002). In addition, the loss of mtHsp70 function in zebrafish recapitulates the ineffective hematopoiesis of the myelodysplastic syndrome (Craven et al., 2005), implicating a role for human mtHsp70 in blood development. The origin of these diverse phenotypes is not yet known. Mitochondrial chaperones have been implicated in a diverse array of cellular processes, including the translocation of nuclear-encoded proteins into the mitochondrial matrix (Matouschek et al., 2000; Neupert and Brunner, 2002; Schneider et al., 1994), the prevention of mitochondrial protein misfolding and aggregation (Kang et al., 1990), the synthesis of Fe-S clusters (Chaudhuri and Paul, 2006; Dutkiewicz et al., 2006; Muhlenhoff et al., 2003; Muhlenhoff et al., 2002), and centrosome duplication (Bukau et al., 2006). Like other hsp70 family members, mtHsp70 is thought to achieve these diverse functions by coupling the binding and release of diverse peptide substrates by its C-terminal domain with cycles of ATP binding and hydrolysis by its N-terminal ATPase domain (Hoff et al., 2000; Silberg et al., 2001; Silberg et al., 2004).

The yeast mitochondrial hsp70 molecular chaperones Ssc1 and Ssq1 require the presence of a specialized escort protein to fulfill their cellular functions. *S. cerevisiae* lacking the hsp70 escort protein Hep1 exhibit a phenotype that mirrors that observed

upon mitochondrial hsp70 chaperone depletion. This includes decreased import of nuclear-encoded proteins into the mitochondrial matrix (Burri et al., 2004; Yamamoto et al., 2005), decreased activities of Fe-S proteins (Sanjuan Szklarz et al., 2005; Sichting et al., 2005), and defects in mitochondrial morphology (Sanjuan Szklarz et al., 2005). These phenotypes are correlated with an accumulation of insoluble Ssc1 and Ssq1, suggesting that Hep1 is needed to maintain these hsp70 chaperones in a soluble conformation (Sanjuan Szklarz et al., 2005; Sichting et al., 2005). Bacterial expression studies have provided evidence that escort proteins play a direct role in enhancing mitochondrial chaperone solubility, as yeast Ssc1 is insoluble when synthesized in *E. coli* unless it is coexpressed with Hep1 (Sichting et al., 2005).

Like Ssc1 and Ssq1, human mtHsp70 is insoluble when overexpressed in bacteria unless it is coexpressed with human mitochondrial Hep (Figure 2.2 and 2.4). Hep is thought to promote mtHsp70 solubility by facilitating the proper folding of the N-terminal ATPase domain of mtHsp70 (70^{ATPase}). 70^{ATPase} is insoluble unless it is coexpressed with Hep (Figure 2.2 and 2.4), whereas the isolated substrate-binding domain is soluble when it is expressed alone (Schilke et al., 2006). Human Hep also stimulates the steady-state ATPase activity of mtHsp70 through an interaction with the ATPase domain (Figure 2.9), suggesting that escort proteins may have a role in regulating the function of folded, nucleotide-bound hsp70s. Currently, the mechanism by which human Hep binds to the different conformational states of mtHsp70 is not known, and it remains unclear if similar residues in Hep are responsible for promoting the solubility of mtHsp70 and stimulating chaperone ATPase activity. The discovery in Chapter 2 that yeast Hep1 can function as an escort protein for human mtHsp70

implicates residues conserved among human and yeast escort proteins as important for escort protein activity.

To better understand human Hep regulation of mtHsp70, I investigated whether charged residues that are conserved in human Hep and yeast Hep1 (25% amino acid sequence identity) are required for regulation of mtHsp70 ATPase activity and solubility by Hep. In addition, I investigated the effects of human Hep expression on the growth defects of $\Delta hep1$ yeast, as well as three Hep mutants.

3.2. Experimental procedures

3.2.1. Materials

E. coli XL1-Blue and Rosetta 2 cells were from Stratagene and EMD Biosciences, respectively, and the *S. cerevisiae* strain was from OpenBiosystem. Enzymes for DNA manipulation were obtained from Roche Biochemical, New England Biolabs, and Promega. Synthetic oligonucleotides were obtained from Fischer Scientific, pET vectors were from EMD Biosciences, and pRS415GPD and pRS416 vectors are from ATCC. NuPAGE Novex 10% Bis-Tris gels from Invitrogen were used for all electrophoresis experiments. Bacterial and yeast growth media components were from BD Biosciences, and gentamicin sulfate (G418) and 5'-fluoroorotic acid (5'-FOA) were from Fisher Scientific. All other reagents were from Sigma-Aldrich.

3.2.2. Vectors

The vectors used for expressing Hep (pHis-Hep), 70^{ATPase} (pATPase-His), and full-length mtHsp70 (pHsp70-His) without mitochondrial targeting sequences were described

in Chapter 2. The gene encoding Hep1 was subcloned from pHep1 into pET30a using NcoI and HindIII to create pHisHep1, a vector that produces *S. cerevisiae* Hep1 without its mitochondrial targeting sequence but with a N-terminal (His)₆ tag that can be removed with Enterokinase. A vector for expressing a tryptophan-free Hep variant (pHis-HepW115I) in *E. coli* was created through Quikchange mutagenesis by introducing a W115I mutation into pHis-Hep, a vector derived from pET30a that expresses Hep with an N-terminal (His)₆ tag that can be removed with enterokinase (*see* Chapter 2). Vectors for expressing tryptophan-free Hep with alanine substitutions at charged residues (K76A, R81A, K88A, H107A, D111A, D118A, E126A, and E134A) were also created through Quikchange mutagenesis of pHis-HepW115I. These vectors are designated pHepK76A, pHepR81A, pHepK88A, pHepH107A, pHepD111A, pHepD118A, pHepE126A, and pHepE134A. A vector expressing Hep-H107A (pHepH107A-WT) was created through Quikchange mutagenesis by removing the W115I mutation in pHepH107A. A gene fragment encoding the residues in Hep (64-160) that are homologous to the trypsin resistant core-domain of *S. cerevisiae* Hep1 (Momose et al., 2007) was PCR amplified for pHis-Hep and cloned into pET30a vector at *ncoI* and *hindIII* sites (pHepCore). Like the vectors for expressing each Hep mutant, this vector produces a Hep truncation with a N-terminal (His)₆ tag that can be removed with enterokinase.

To create a vector that supports growth of a haploid $\Delta hep1$ strain in the absence of 5'-FOA, the *hep1* gene and its promoter were PCR amplified from yeast genomic DNA (chromosome 14, region 52661 to 51687) and cloned into the centromeric plasmid pRS416 (Sikorski and Hieter, 1989) using *xbal* and *hindIII* to create pURA-ScHep. To create vectors that could be used for complementation analysis, full-length human Hep

and yeast Hep1 were PCR amplified from pHep-EGFP and a *S. cerevisiae* genomic DNA (BY4743, OpenBiosystem) and cloned into the centromeric yeast plasmid pRS415GPD (Mumberg et al., 1995) using *xbal*I and *hind*III restriction sites to create vectors that use a GPD promoter to constitutively express Hep (p415HsHep) and Hep1 (p415ScHep). Quikchange mutagenesis was used with each of these plasmids to create yeast expression vectors that constitutively express *S. cerevisiae* Hep1-H107A (p415-ScHep-H107A) and human Hep-H107A (p415HsHep-H107A), Hep-R81A (p415HsHep-R81A), and Hep-K88A (p415HsHep-K88A). All constructs were sequence verified.

3.2.3. Protein Purification

Recombinant Hep (pHis-Hep), mtHsp70 (pHsp70-His), and 70^{ATPase} (pATPase-His) lacking mitochondrial targeting sequences were expressed and purified as previously described in Chapter 2. Each Hep mutant was purified using a procedure similar to that reported for Hep. Rosetta 2 (DE3) cells transformed with pET vectors that express each mutant were grown at 37°C, induced to express protein with 0.1 mM isopropylthio- β -D-galactoside (IPTG) at an $A_{600} \cong 1$, and grown for ≈ 18 hours at 23°C to allow for expression. Harvested cells were resuspended in TND buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM DTT) containing 1 mM MgCl₂, 300 μ g/ml lysozyme, and 2 units/ml DNase I. After two cycles of freezing at -80°C and thawing, lysed cells were centrifuged at 40,000 x g to remove cell debris. Cleared lysate was applied to HisTrap column (GE Healthcare), the column was washed with TND buffer containing 15 mM imidazole to remove unbound protein, and His-tagged variants were eluted with TND buffer containing 150 mM imidazole. After dialysis against TKMD buffer (50 mM Tris

pH 8.0, 150 mM KCl, 10mM MgCl₂, 1mM DTT) containing 100 μ M ZnCl₂, fusion protein (~10 mg/ml) was treated with 1 unit/ml enterokinase (EMD Bioscience) for 72 h at 23°C and applied to Histrap column to remove the affinity tag and uncleaved protein. The flow-through fractions were concentrated and chromatographed using a S75 Superdex column (GE Healthcare) in TKMD buffer containing 30 μ M ZnCl₂, and fractions appearing homogeneous were pooled and concentrated to ≥ 300 μ M. ZnCl₂ was included in all buffers during purification because it inhibited protein aggregation.

3.2.4. Escort activity screen

Rosetta 2 *E. coli* transformed with pHsp70 in the absence and presence of vectors that express Hep variants were grown at 37°C in LB to mid-logarithmic phase, induced to express proteins by adding 0.1 mM isopropylthio- β -D-galactoside (IPTG), and grown for ~18 hours at 23°C. Cells were harvested by centrifugation, resuspended in TND buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT) containing 1 mM MgCl₂, 300 μ g/mL lysozyme, 2 U/mL DNase I, and frozen at -80°C overnight. Lysed cells were thawed, the total protein concentration was determined using Bio-Rad Protein Assay, and the lysate was centrifuged at 40k x g to separate the insoluble and soluble proteins. The insoluble fractions were resuspended in TND buffer to the same volume as the soluble fraction. Equal volumes of total, soluble, and insoluble fractions corresponding to 10 μ g soluble protein per sample were analyzed by SDS-PAGE.

3.2.5. Fluorescence analysis

Emission spectra of the mtHsp70 ATPase domain (2 μ M) were acquired in the absence and presence of increasing levels of each tryptophan-free Hep mutant (0 to 40 μ M) in TKMD buffer at 25°C using an excitation wavelength of 285 nm. To account for internal absorption, the fluorescence at each Hep concentration (\pm chaperone) was calculated as $F_{\text{corrected}} = F_{\text{obs}} \cdot \text{antilog}(\text{OD}_{\text{ex}} + \text{OD}_{\text{em}})/2$, where OD_{ex} and OD_{em} are the absorption of Hep mutants at the excitation and emission wavelength at each concentration (Lakowicz, 2006), and the change in chaperone fluorescence for each condition was calculated by subtracting the $F_{\text{corrected}}$ of each Hep mutant from the $F_{\text{corrected}}$ of the 70^{ATPase}/Hep mixtures. To determine the fraction of Hep bound to 70^{ATPase}, the fluorescence quenching at 340 nm was plotted as a function of escort protein concentration, and the data were fit to a simple binding model that assumes 1:1 stoichiometry. The error bars represent \pm one standard deviation.

3.2.6. Copurification of protein complexes

Hep variants (20 μ M) were incubated with His-tagged mtHsp70 ATPase domain (20 μ M) in 200 μ L TKMD buffer at 23°C for 15 minutes. Each protein mixtures was combined with 20 μ L of NTA resin pre-equilibrated in TKMD, incubated for 15 minutes, and washed with TKMD buffer containing 5mM imidazole to remove unbound protein. NTA-bound protein was eluted through addition of SDS-PAGE loading buffer prior to PAGE.

3.2.7. ATPase assays

Steady state ATP hydrolysis rates were measured using the EnzCheck phosphate detection kit (Invitrogen) as previously described (Silberg et al., 2004; Silberg and Vickery, 2000). First order rates were measured in TKMD buffer (pH 8.0) or HKMD (50 mM HEPES pH 7.4, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT). Reactions were performed by incubating all components except ATP for 5 minutes at 25°C and then initiating reactions by adding ATP to a final concentration of 1 mM. The curves shown represent least-squares fits of the data to Michaelis-Menten equation. The error bars represent \pm one standard deviation.

3.2.8. Surface plasmon resonance

The mtHsp70 ATPase domain was cross-linked to a CM5 sensor chip (GE Healthcare) in 10 mM sodium acetate (pH 5.0) containing 1 mM MgCl₂ and 1 mM ADP via amine coupling using a Biacore T-100. Hep-W115I and Hep-W115I/H107A (40 μ M) were injected for 120 seconds at a flow rate of 60 μ l/min in TKMD (pH 8.0) or HKMD (7.4) running buffer containing 0 or 1 mM ATP (or ADP).

3.2.9. Zinc assay

Hep and Hep-W115I/H107A (0 to 10 μ M) were incubated in 50 mM HEPES pH 7.5 with 100 μ M 4-(2-pyridylazo) resorcinol (PAR) and 200 μ M methyl methanethiolsulphonate (MMTS) for 60 minutes. The PAR-(Zn²⁺)₂ complex was detected by measuring the absorbance at 500 nm using a Cary-50 UV-Vis spectrophotometer. The amount of zinc released was calculated using an extinction coefficient for PAR-(Zn²⁺)₂ at

500 nm of $68,403 \text{ M}^{-1}\text{cm}^{-1}$ that was generated using a ZnCl_2 standard curve.

Measurements were performed in triplicate at three different protein concentrations, subtracted by the level of zinc found in buffer against which Hep1 was dialyzed, and are reported ± 1 standard deviation.

3.2.10. Yeast complementation

The *S. cerevisiae* heterozygous strain YSC1021-669052 (Winzeler et al., 1999) having one of its chromosomal *hep1* genes replaced by *kanMX* (*MATa/a his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 LYS2/lys2 Δ 0 met15 Δ 0/MET15 ura3 Δ 0/ura3 Δ 0 HEP1::KanMX/HEP1*) was transformed with pURA-ScHep using the EZ Yeast Transformation II kit (Zymo research), and a haploid strain (Δ *hep1*/pURA-ScHep) was obtained by sporulation followed by tetrad dissection using random spore isolation method (Rockmill et al., 1991). The isolated spores were grown on agar plates containing synthetic complete dextrose (SCD) medium (-uracil) and 300 $\mu\text{g/mL}$ G418. To screen for *met15 Δ 0* haploids, colonies were patched to agar plates containing complete or -methionine SCD and 300 $\mu\text{g/mL}$ G418.

The haploid Δ *hep*/pURA-ScHep strain was transformed with centromeric vectors encoding each Hep variant using Frozen-EZ Yeast transformation II kit (Zymo Research), and transformed cells were grown on agar plates containing -leucine drop-out medium and 300 $\mu\text{g/mL}$ G418. Colonies harboring plasmids that express human Hep were identified by PCR screening with primers that amplify the human Hep. Escort protein complementation of Δ *hep*/pURA-ScHep was analyzed by growing colonies in SCD liquid medium (-uracil/-leucine) containing 300 $\mu\text{g/mL}$ G418 to an optical density ~ 2 ,

plating 10 μ L dilutions of this culture (corresponding to an OD of 1, 0.1, 0.01, and 0.001) on agar plates containing SCD medium \pm 1 mg/mL 5'-FOA, and imaging growth after 48 hours (0 mg/mL 5'-FOA) and 96 hours (1 mg/ml 5'-FOA) using a digital camera. The previously described haploid Δ *hep1* strain (Blamowska, 2010; Sichtung et al., 2005) available from Euroscarf (#10179B) was not used because its growth could not be complemented by p415ScHep.

3.2.11. Analytical methods

An ÄKTA FPLC was used for all protein purification, absorbance measurements were performed using a Cary 50 spectrophotometer, and fluorescence and circular dichroism analysis was performed using a JASCO 815 spectropolarimeter.

3.3. Results

3.3.1. A tryptophan-free Hep mutant

The mtHsp70 ATPase domain contains a single tryptophan (Trp115) whose intrinsic fluorescence is expected to be sensitive to substrate and cochaperone binding as observed with other molecular chaperones (Buchberger et al., 1995; Reid and Fink, 1996; Slepnev and Witt, 1998). To investigate whether changes in the tryptophan fluorescence of 70^{ATPase} occur upon binding to Hep, I created a tryptophan-free Hep mutant lacking its predicted mitochondrial targeting sequence (residue 50 to 174) and examined the effect of this protein on 70^{ATPase} fluorescence. The single tryptophan in this portion of Hep was replaced with the homologous isoleucine in yeast Hep1 (Figure 3.1) to minimize the effects of this mutation on protein function. The effect of Hep-W115I on

```

Hs  MLRTALRGAPRLLSRVQPRAPCLRRLLWGRGARPEVAGRRRAWAWGWRSSSEQGP--GPA
Sc  ----MIPRTRTLLQSKIPITRYFARCWAPRVRYNVCRTLPAALHTNIIAHNEVKDDKK
      . .  ::   : . . : :   : :   : :   Δ   . . .
Hs  AALG--RVEAAHYQLVYTCKVCGTRSSKRISKLAYHQQGVVIVTCPGCQNHHIADNLGWF
Sc  VHLGSFKVDKPKMMIAFTCKKCNTRSSHTMSKQAYEKGTVLISCPHCKVRHLIADHLKIF
      ::  . . . . . : : : : : . : : : : : : : : : : : : : : : :
Hs  SDLNGKRNIEEILTARGEQVHRVAGEGALELVLEAAGAPTSTAAPAEAGEDEGPPSPGKTEPS
Sc  HDHH--VTVEQLMKANGEQVSQDVGDLFEDIPDSLKDVLGKYAKNNSENASQLPHPSQK--
      : .   . : : : : .   . .   . .   . .   . .   . .   .

```

Figure 3.1. Alignment of the sequences of yeast Hep1 and human Hep showing conserved charged residues. Identical (:) and similar (.) amino acids are indicated. The conserved residues targeted for alanine mutation are shown in bold type (K76, R81, K88, H107, D111, D118, E126, and E134). In addition, the tryptophan in Hep that was mutated to isoleucine (*) and the locations of mitochondrial presequence processing (Δ) are indicated.

70^{ATPase} fluorescence is shown Figure 3.2. Nucleotide-free 70^{ATPase} (2 μM) exhibits a steady-state fluorescence spectrum with maximal emission at 340 nm upon excitation at 285 nm that is ~ 5 -fold higher in intensity than Hep-W115I (20 μM). In contrast, the steady-state emission of a 70^{ATPase} (2 μM) and Hep-W115I (20 μM) mixture is $\sim 20\%$ lower than that of 70^{ATPase} alone. The effect of increasing levels of Hep-W115I on 70^{ATPase} emission is shown in Figure 3.3. The quenching of 70^{ATPase} fluorescence can be described by a simple 1:1 binding model assuming an equilibrium binding constant (K_D) of 5 μM and maximal quenching of 26.6% (relative to that of Hep-W115I and 70^{ATPase} before mixing).

To evaluate whether the tryptophan mutation causes a functional defect in Hep, I examined whether Hep-W115I stimulates 70^{ATPase} activity and promotes the solubility of newly synthesized mtHsp70. Figure 3.4 compares the effects of Hep and Hep-W115I on the solubility of recombinant full-length mtHsp70 overexpressed in *E. coli*. As previously observed, IPTG-induced expression of mtHsp70 leads to a major band at ~ 70 kDa that is found within the insoluble fraction. In contrast, mtHsp70 is found primarily within the soluble fraction when it is coexpressed with Hep-W115I or Hep. Figure 3.5 compares the effects of increasing concentrations of Hep and Hep-W115I on the steady-state ATPase activity of 70^{ATPase} under conditions that reflect the resting pH (8.0) of mitochondria (Llopis et al., 1998). The activity of 70^{ATPase} was stimulated by both proteins, and similar concentrations of Hep and Hep-W115I were required to elicit half-maximal stimulation ($K_M \cong 4 \mu\text{M}$). However, the maximal stimulation caused by Hep-W115I (23.1-fold) was greater than that of Hep (12.6-fold). These findings show that the

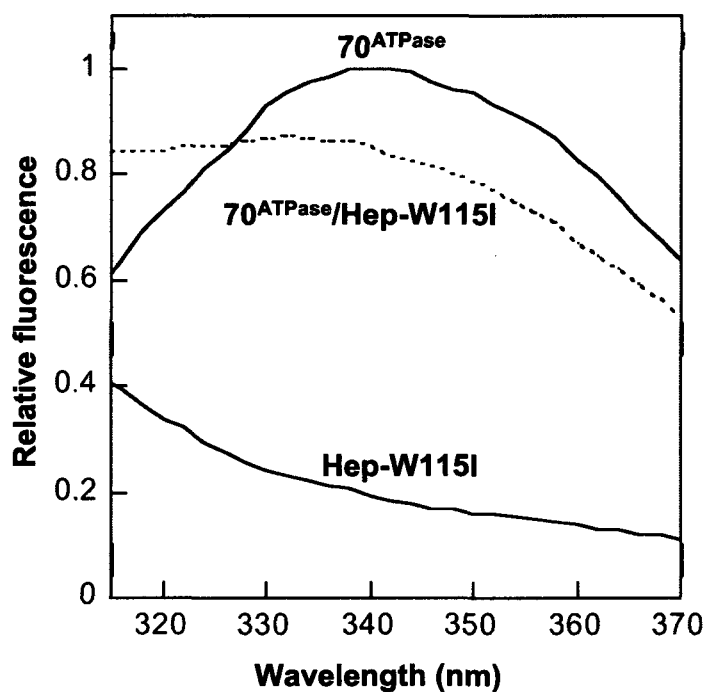


Figure 3.2. Hep-W115I decreases 70^{ATPase} tryptophan fluorescence.

Emission spectra of 70^{ATPase} (2 μ M), Hep-W115I (20 μ M), and a mixture of 70^{ATPase} (2 μ M) and Hep-W115I (20 μ M) were recorded in TKMD (pH 8.0) buffer at 25 °C. The fluorescence of mtHsp70 in the presence of Hep-W115I (dashed line) was calculated by subtracting the spectrum acquired for Hep-W115I alone from the spectrum of the mixture.

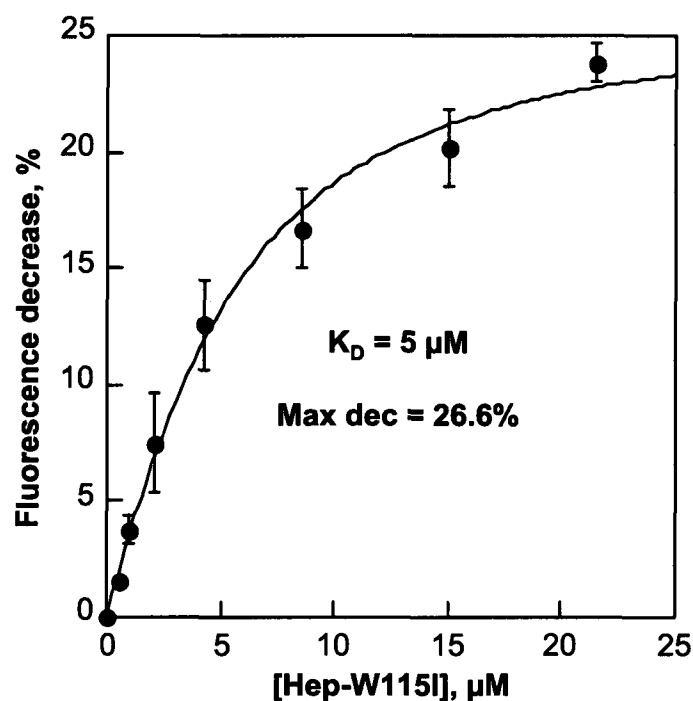


Figure 3.3. Effect of increasing concentrations of Hep-W115I on the fluorescence of 70^{ATPase} . Experiments were performed using 0 to 20 μM Hep and 2 μM 70^{ATPase} in TKMD (pH 8.0) buffer at 25 °C. The fluorescence values shown represent measured values for mixtures of 70^{ATPase} and Hep-W115I subtracted by the value measured for Hep alone and the calculated inner filter effect arising from the absorbance of Hep-W115I. The curve shown represents a best fit of the data to quadratic function with a maximal quenching of 27% and half-maximal quenching at 5 μM free Hep-W115I.

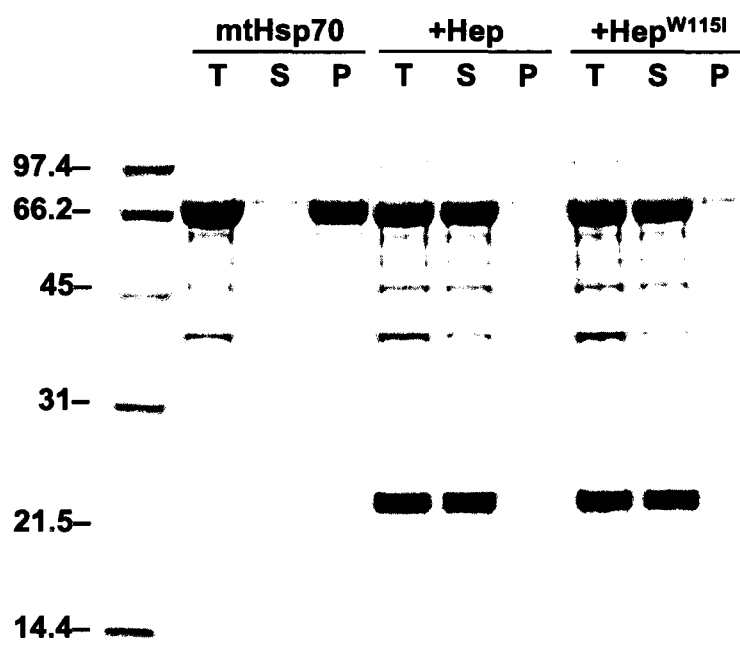


Figure 3.4. Effect of W115I mutation on escort activity. SDS-PAGE gel analysis of Hep-W115I escort activity. Molecular weight marker (lane 1), Rosetta 2 *E. coli* harboring a plasmid that expresses mtHsp70 alone (lane 2-4), and with wild-type Hep (lane 5-7) or Hep-W115I (lane 8-10). For each sample, 10 μ g of total protein from whole cells (T) is shown as well as the soluble lysate (S) and pellets (P) derived from a sample that contained the same amount of protein prior to fractionation.

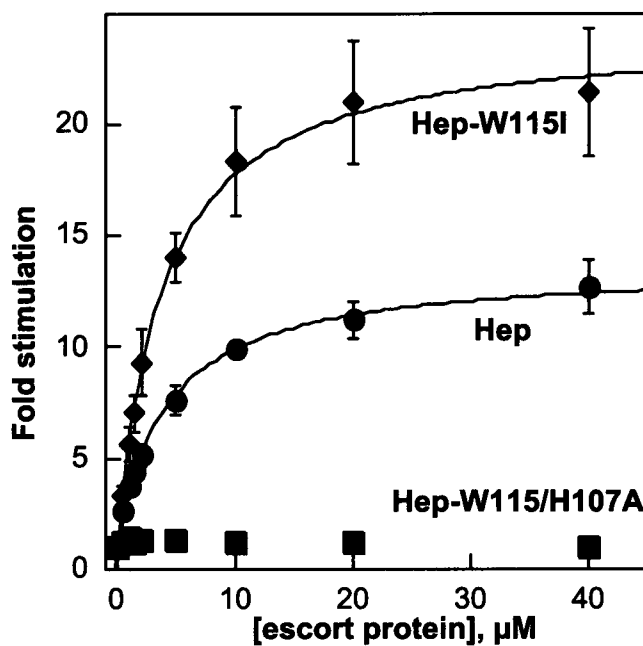


Figure 3.5. Effect of W115I and H107A mutations on Hep stimulation of 70^{ATPase} activity. The effect of increasing concentrations (0 to 40 μM) of Hep (circles), Hep-W115I (diamonds), and Hep-W115I/H107A (squares) on the ATPase activity of 70^{ATPase} (1 μM) in TKMD buffer containing 1 mM ATP at 25 °C. The curves shown represent hyperbolic saturation functions, assuming a maximal stimulation of 12.6- (Hep) and 23.1-fold (Hep-W115I) with half-maximal stimulation at total escort protein concentrations of 4.1 and 3.8 μM , respectively. All reaction components were mixed and incubated at 25 °C for 5 minutes prior to starting the reactions through the addition of ATP.

W115I mutation does not abrogate Hep escort activity but does enhance its ability to stimulate chaperone ATPase activity.

3.3.2. Hep mutant effects on 70^{ATPase} fluorescence

The experiments described in Chapter 2 found that yeast Hep1 promotes the solubility of human mtHsp70 upon overexpression in *E. coli*, like human Hep. In addition, Figure 3.6 shows that yeast Hep1 stimulates 70^{ATPase} steady-state activity up to 3.5-fold with half-maximal stimulation at a concentration (6.6 μM) similar to that required for half-maximal stimulation by Hep (4.1 μM). Taken together, these findings implicate a role for residues conserved among yeast and human escort proteins in regulating mtHsp70 solubility and catalytic activity. To determine the relative contribution of these conserved residues to escort protein functions, I carried out an alanine mutagenesis scan of charged residues in human Hep that are conserved in the trypsin-resistant core of Hep1, a region of Hep1 that is functional in yeast (Momose et al., 2007). Efforts focused on these residues, since a truncation of human Hep (residues 64 to 160) homologous to this fragment is capable of stimulating chaperone ATPase activity (*see* Table 3.1). All alanine mutations were created within Hep-W115I to allow for fluorescent analysis of chaperone binding.

Figure 3.7A shows the effects of increasing concentrations of the different Hep mutants on the fluorescence of 70^{ATPase} . Like Hep-W115I, which quenches 70^{ATPase} fluorescence up to 27%, five of the alanine mutants quench chaperone fluorescence by >20% when present at a concentration (40 μM) that is 8-fold higher than equilibrium binding constant measured for Hep-W115I and 70^{ATPase} . This includes the variants

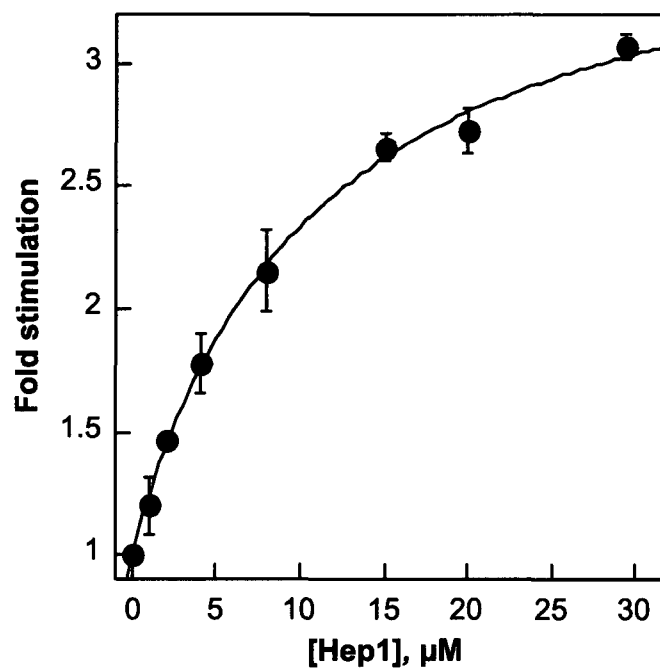


Figure 3.6. Yeast Hep1 stimulation of human mtHsp70 ATPase activity. The effect of Hep1 (0 to 30 μM) on 70^{ATPase} (1 μM) in TKMD buffer containing 1 mM ATP at 25° C. A fit of the Michaelis-Menten Equation to the data for Hep1 yields a maximal stimulation of 3.5 fold with half-maximal stimulation at 6.6 μM Hep1.

Hep mutations	V_{max}	K_M (μ M)
Hep	12.6	4.1
W115I	23.1	3.8
64-160	9.9	3.4
W115I/K76A	6.7	6.8
W115I/R81A	6	38
W115I/K88A	11	18
W115I/H107A ¹	0	---
W115I/D111A	5.4	6
W115I/D118A	5.8	11
W115I/E126A	9.8	16.9
W115I/E134A	8.3	15
H107A ²	0	---

Table 3.1. Stimulation of 70^{ATPase} activity by escort proteins. The effect of increasing concentrations of Hep mutants (0 to 40 μ M) on the steady-state ATPase activity of 70^{ATPase} (1 μ M) were determined in TKMD (pH 8.0) buffer at 25 °C. The maximal stimulation (V_{max}) and concentration required for half-maximal stimulation (K_m) were determined by fitting the data to a 1:1 binding model as described (Silberg et al, 2004; Silberg et al 2000).

¹ Does not stimulate full-length mtHsp70 under conditions where Hep (40 μ M) elicits a 37-fold increase.

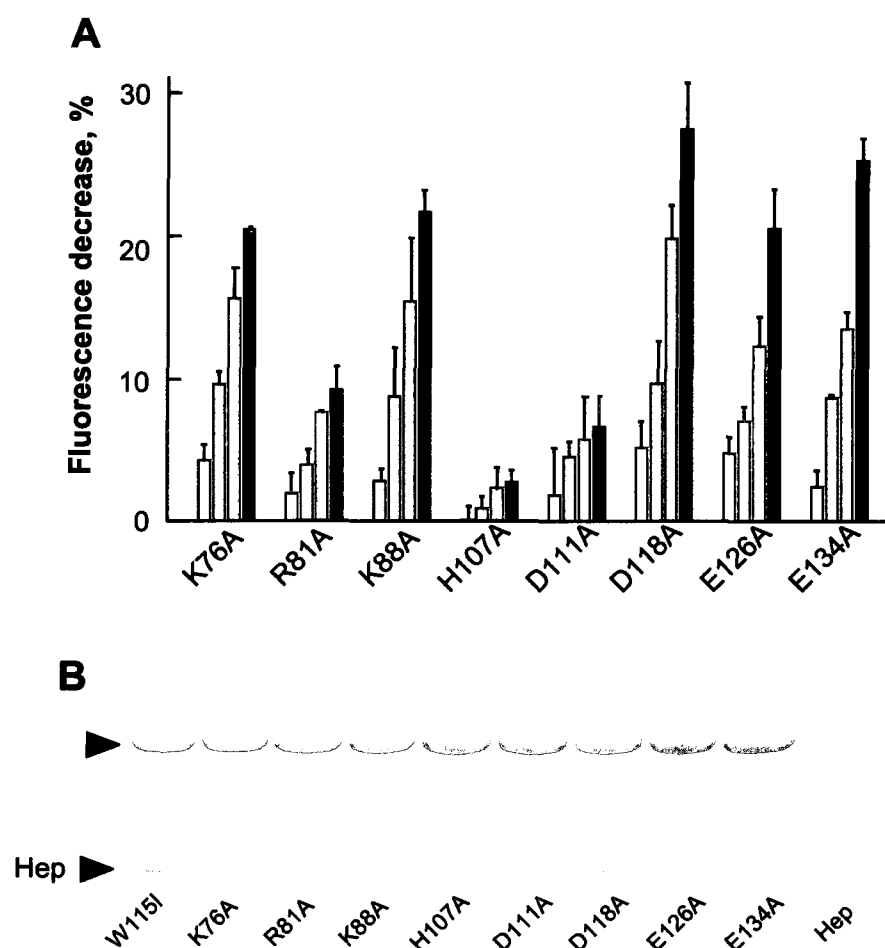


Figure 3.7. Effects of mutations on Hep binding to nucleotide-free 70^{ATPase}.
A, Percentage change in the tryptophan fluorescence of 2 μ M 70^{ATPase} in the presence of 2, 5, 15, and 40 μ M Hep-W115I lacking or harboring K76A, R81A, K88A, H107A, D111A, D118A, E126A, and E134A mutations. *B*, SDS-PAGE analysis of Hep mutants that copurify with His-tagged 70^{ATPase}. Mixtures containing His-tagged 70^{ATPase} (20 μ M) and the indicated variants of Hep (20 μ M) were incubated in TKMD buffer for 15 minutes at room temperature, applied to Ni-NTA resin, and washed with excess buffer. Bound protein was eluted by addition of SDS-loading buffer and analyzed by PAGE. The last lane represents the results from an experiment using Hep-W115I alone (20 μ M).

harboring the K76A, K88A, D118A, D126A, and E134A mutations. In contrast, three of the alanine mutants quenched 70^{ATPase} fluorescence by <10% when present at a similar concentration, including the R81A, H107A, and D111A mutants. H107A elicited the smallest change in 70^{ATPase} fluorescence (2.7%), suggesting that this mutation causes the largest defect in chaperone binding in the absence of nucleotide.

To test whether changes in the magnitude of fluorescence quenching reflect a decrease in binding affinity of 70^{ATPase} for the Hep mutants, I used affinity purification to evaluate the effects of each mutation on the stability of the Hep- 70^{ATPase} complex. Figure 3.7B shows the relative levels of each Hep mutant (20 μM) that associate with NTA resin after incubation with equimolar His-tagged 70^{ATPase} . Similar amounts of the K76A, K88A, D118A, D126A, and E134A copurified with 70^{ATPase} as Hep-W115I. In contrast, the amount of the R81A, H107A, and D111A mutants that copurified with 70^{ATPase} were decreased compared to W115I alone and similar to the level of Hep-W115I observed when experiments were performed in the absence of His-tagged 70^{ATPase} .

3.3.3. Hep mutant effects on 70^{ATPase} activity

In Chapter 2, I showed that the stability of the mtHsp70 and Hep complex was decreased in the presence of ADP and ATP (Figure 2.7 and 2.8). This suggests that the mechanism of Hep binding to the different conformational states of mtHsp70 may differ. To test this, I examined whether Hep mutants with impaired binding to nucleotide-free 70^{ATPase} had similar defects in their ability to interact with ATP-bound 70^{ATPase} and stimulate ATPase activity. The effect of each Hep mutant on chaperone activity is given in Table 1. All of the mutants except the Hep-W115I/H107A double mutant (*see* Figure

3.5) stimulated 70^{ATPase} activity when present at a concentration (40 μM) that is 10-fold higher than that required for half-maximal stimulation of 70^{ATPase} activity by Hep (and Hep-W115I). The two mutants (R81A and D111A) that displayed defects in binding to nucleotide-free chaperone differed in their regulation. Both mutants stimulated 70^{ATPase} activity to a similar maximal extent (5.4 and 6-fold). However, the concentration of Hep-W115I/R81A required for half-maximal stimulation (38 μM) of 70^{ATPase} was 3.5-fold higher than Hep-W115I/D111A (11 μM) and ~10-fold higher than Hep (and Hep-W115I). A Hep mutant having only the H107A substitution did not stimulate 70^{ATPase} activity when present at a concentration of 40 μM .

3.3.4. Escort activity of Hep mutants

Studies comparing the *in vivo* function of yeast Hep1 mutants with their escort activity in *E. coli* have observed a correlation between loss of function in living cells and lack of escort activity in bacteria (Momose et al., 2007). To test if Hep variants with *in vitro* binding defects display escort activity, I compared the effect of each Hep-W115I alanine mutant on full-length mtHsp70 solubility upon overexpression in *E. coli*. Figure 3.8 compares the soluble fraction of cells expressing mtHsp70 alone and in the presence of the different Hep-W115I alanine mutants. In all cells harboring vectors for mtHsp70 and a Hep mutant, two major bands were observed that were not seen when mtHsp70 was expressed alone. These bands exhibit a molecular weight and intensity consistent with that observed previously for recombinant Hep and mtHsp70 (Figure 2.4), indicating that all eight mutants including the Hep-W115I/H107A are capable of promoting mtHsp70 under the conditions of this assay.

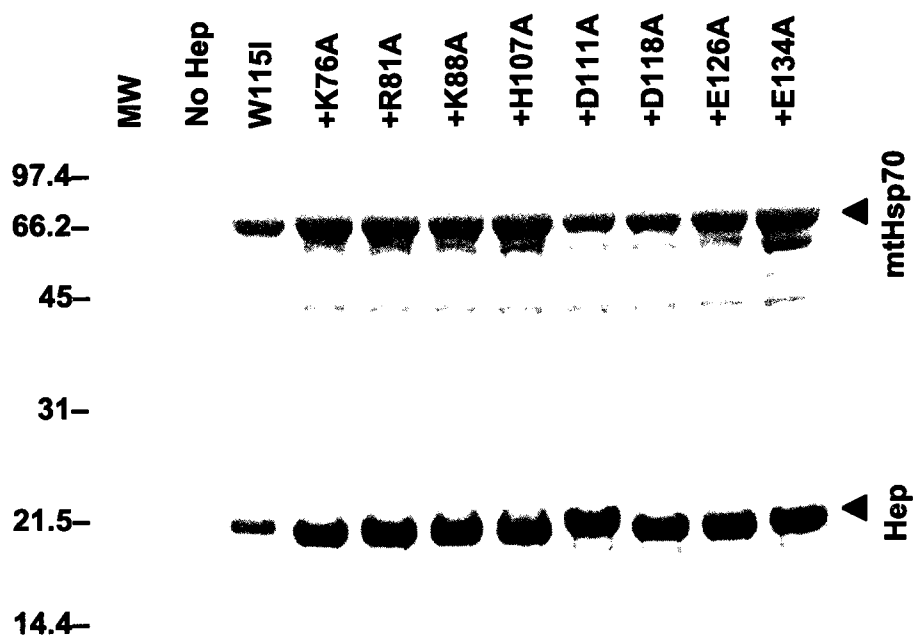


Figure 3.8. SDS-PAGE analysis of Hep mutant escort activity. The soluble fraction (10 μ g) of *E. coli* overexpressing full-length mtHsp70 in the absence and presence of Hep-W115I variants harboring mutations within charged residues.

3.3.5. SPR analysis of binding

The finding that Hep-W115I/H107A exhibits escort activity within *E. coli* which has a cytosolic pH (Wilks and Slonczewski, 2007) that is lower than that of mitochondria (Llopis et al., 1998), suggested that the interaction of this mutant with 70^{ATPase} may be sensitive to decreases in pH. To test this, surface plasmon resonance (SPR) analysis was used to investigate the effect of the H107A mutation on the interaction of Hep-W115I with mtHsp70 in different buffers. Figure 3.9A shows the results of titrations in which 70^{ATPase} was immobilized to the surface of a CM5 sensor chip and then exposed to Hep-W115I or Hep-W115I/H107A (40 μM) in buffer whose pH (8.0) reflects that within resting mitochondria (Llopis et al., 1998). Under these conditions, Hep-W115 binding to 70^{ATPase} was observed in the presence and absence of ADP (and ATP), with the largest signal arising in the absence of nucleotide. However, the signal arising from Hep-W115I/H107A was $\leq 10\%$ of that observed with Hep-W115I in both the presence and absence of nucleotides. SPR measurements were repeated with a similar escort protein concentration at a pH (7.4) that more closely reflects that of the *E. coli* cytosol in *E. coli* where our escort activity assay was performed (Wilks and Slonczewski, 2007). Figure 3.9B shows that under these conditions the signal arising from Hep-W115I binding is similar to that observed at pH 8.0 (\pm nucleotides). In contrast, the Hep-W115I/H107A double mutant binds immobilized 70^{ATPase} to a greater extent at this pH when ADP or ATP are present in the buffer, although there was no increase in the binding of Hep-W115I/H107A to 70^{ATPase} in the absence of nucleotide. Because Hep-W115I/H107A exhibits enhanced binding to immobilized 70^{ATPase} at pH 7.4 in the presence of ATP, I also measured the effect of this mutant on chaperone ATPase activity under similar

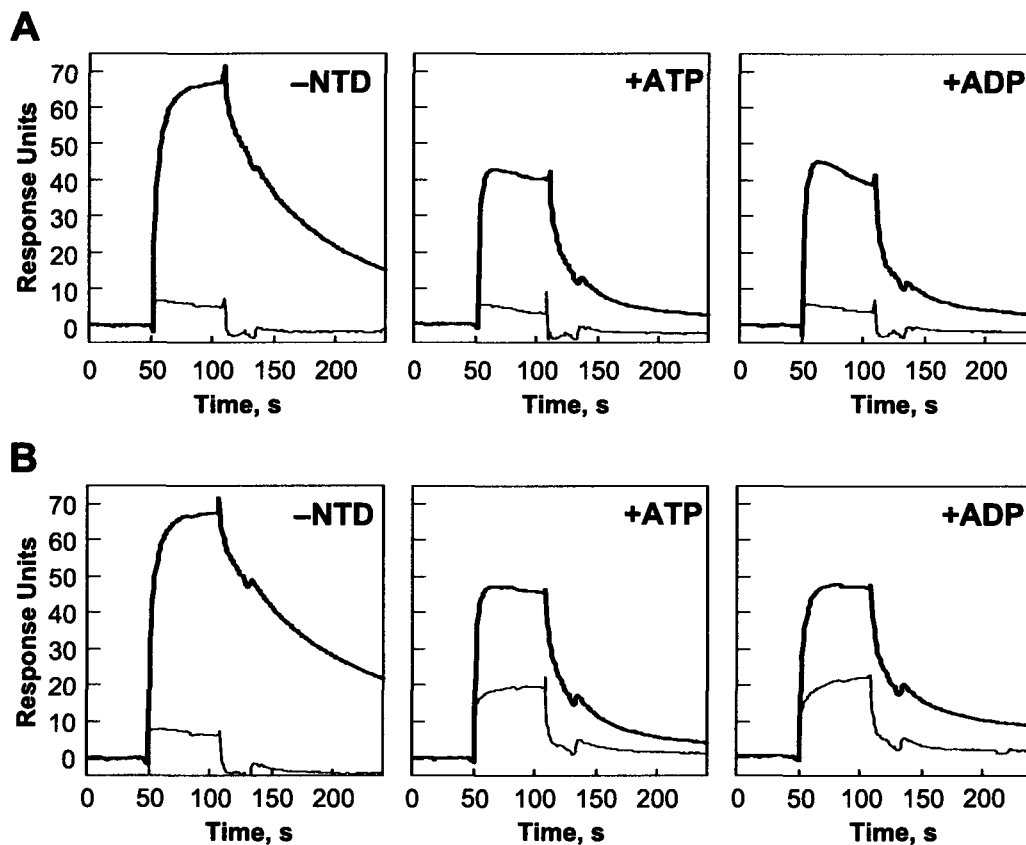


Figure 3.9. SPR analysis of chaperone and escort protein binding. *A*, Hep-W115I and Hep-W115I/H107A (40 μ M) were injected into TKMD buffer (pH 8.0) passing over a sensor chip containing immobilized 70^{ATPase} (317 RU) at 25 $^{\circ}$ C. *B*, Injections were performed with a HKMD buffer (pH 7.4) passing over the sensor chip. Nucleotide concentrations were 1 mM.

conditions. Addition of 40 μ M Hep-W115I/H107A (or Hep-H107A) to 70^{ATPase} did not enhance chaperone activity, similar to that observed at pH 8.

3.3.6. Zinc content of Hep

To examine whether the H107A mutation affects zinc binding to Hep, Hep-W115I/H107A lacking a His tag was mixed with a >10-fold excess of MMTS, a reagent that reacts with sulfhydryl groups (Smith et al., 1975), and the level of zinc released was monitored using PAR, a zinc chelator that exhibits increased absorbance at 500 nm upon zinc binding (Hunt et al., 1985). When MMTS was added in the presence of excess PAR, the absorbance increased to a level that corresponded with 1.09 ± 0.02 equivalents of zinc released per Hep-W115I/H107A. Similar experiments performed with Hep-W115I revealed the presence of 1.24 ± 0.01 equivalents of zinc per Hep. Spectropolarimetry analysis further revealed that the W115I and H107A mutations do not affect the secondary structure of Hep (Figure 3.10).

3.3.7. Human Hep complements yeast Δ hep1

Human *hep* was cloned into a yeast shuttle vector, and the ability of this plasmid to rescue the growth defect of Δ hep1 *S. cerevisiae* was compared with a vector expressing yeast Hep1. Figure 3.11 shows that yeast lacking a functional Hep1 are not viable on medium containing 5'-FOA, similar to that previously reported (Momose et al., 2007; Yamamoto et al., 2005). However, cells expressing human Hep grow readily under these conditions, albeit to a lesser extent than cells expressing yeast Hep1. I also constructed vectors for expressing human Hep with the R81A, K88A, and H107A mutations.

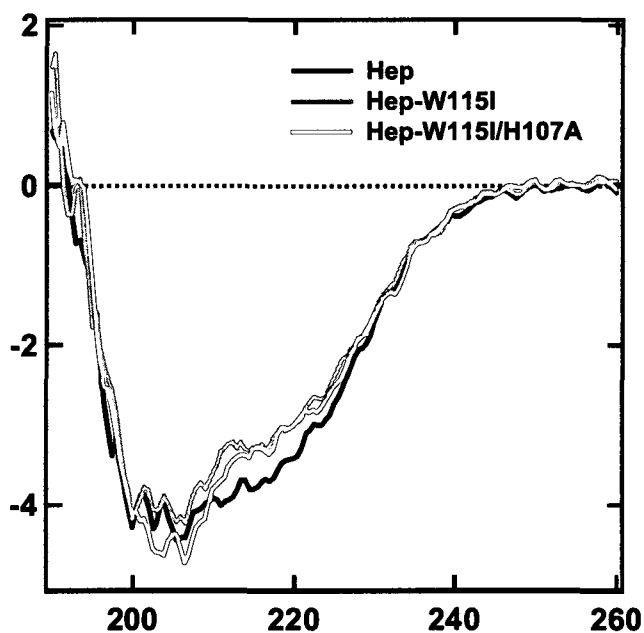


Figure 3.10. Far-UV circular dichroism spectra of Hep variants. CD spectra of Hep (black), Hep-W115I (blue), and Hep-W115I/H107A (red) (10 μ M) were acquired at 25 $^{\circ}$ C in 10 mM potassium phosphate pH 8.0 containing 1 mM DTT. Spectra are reported on a mean residue basis and are corrected for ellipticity of the buffer. All three Hep variants produce near identical CD spectra, which lack the intense minima at the wavelengths where α -helix (208 and 222 nm) and β -sheet (215nm) secondary structure produce the largest negative ellipticity, and exhibit a minimum at 206 nm.

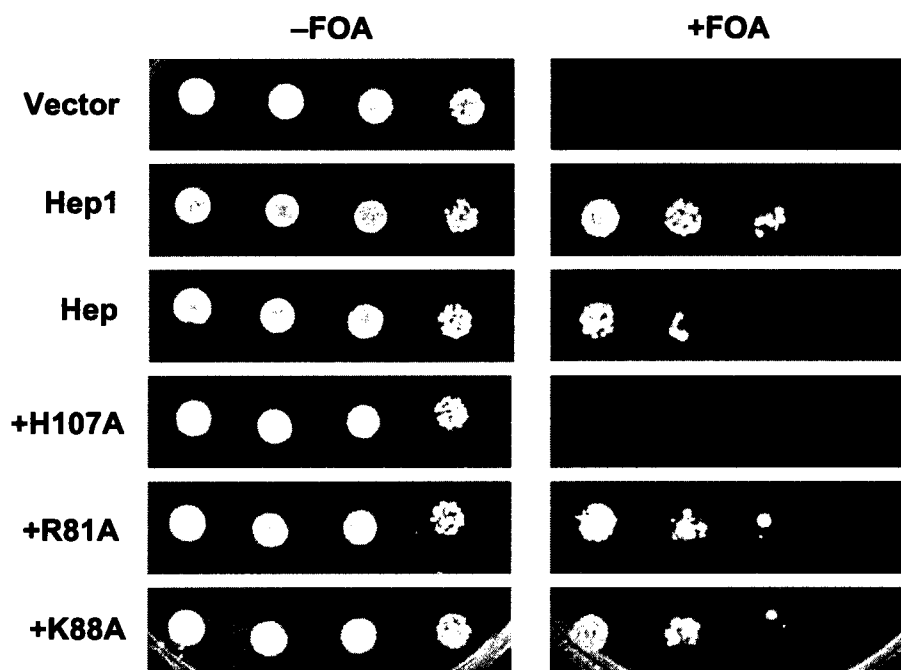


Figure 3.11. Hep complementation of yeast $\Delta hep1$. Relative growth at 30 °C of a $\Delta hep1/pURAScHep$ yeast haploid strain transformed with the p415GPD lacking an insert (vector) and p415GPD-derived vectors that expresses Hep1 (Hep1), Hep (Hep), and Hep mutants harboring alanine substitutions at R81 (HepR81A) and H107 (HepH107A). The spots represent a dilution series of cells (1x, 10x, 100x, and 1,000x) aliquoted onto SCD plates lacking (-FOA, 48 hrs) or containing 5'-FOA (+FOA, 96 hrs), which were obtained from SCD liquid cultures (-uracil/-leucine) containing 300 μ g/mL G418.

Whereas the vectors that express Hep-R81A and Hep-K88A both support $\Delta hep1$ cell growth in the presence of 5'-FOA, the vector that expresses Hep-H107A could not support growth under these conditions.

3.3.8. Escort activity of human Hep truncation mutants

To determine minimum functional fragment of human Hep, I mutated the codons for residues 123, 113, and 106 to stop codons in pET30a-Hep⁶⁴⁻¹⁶⁰. The ensuing vectors express human Hep truncations containing residues 64-122 (pHep⁶⁴⁻¹²²), 64-112 (pHep⁶⁴⁻¹¹²), and 64-105 (pHep⁶⁴⁻¹⁰⁵). These vectors were cotransformed with pHsp70 into Rosetta 2 *E. coli* and their effect on the solubility of full-length mtHsp70 were examined. Figure 3.12 shows that mtHsp70 is found within the soluble fraction when coexpressed with Hep⁶⁴⁻¹⁶⁰ and Hep⁶⁴⁻¹²². In contrast, Hep⁶⁴⁻¹¹² and Hep⁶⁴⁻¹⁰⁵ could not promote the solubility of mtHsp70. This finding suggests that the 59-residue fragment (64-122) represents a new minimal functional unit of human Hep.

3.3.9. Zn²⁺ is required for the solubility of yeast Hep1.

To determine if Zn²⁺ is essential for the solubility of yeast Hep1, I expressed yeast Hep1 in *E. coli* growing in minimum medium (M9) containing different concentrations of zinc chloride. Figure 3.13 shows that *S. cerevisiae* Hep1 is expressed to similar extent in media containing or lacking supplemental zinc. However, Hep1 is predominantly within the insoluble fraction when expressed in the media without Zn²⁺, while it soluble in the media with 1 mM Zn²⁺. This suggests that Zn²⁺ is essential for the solubility of yeast Hep1. To determine whether Zn⁺² is required for the solubility of Hep1 after folding, I

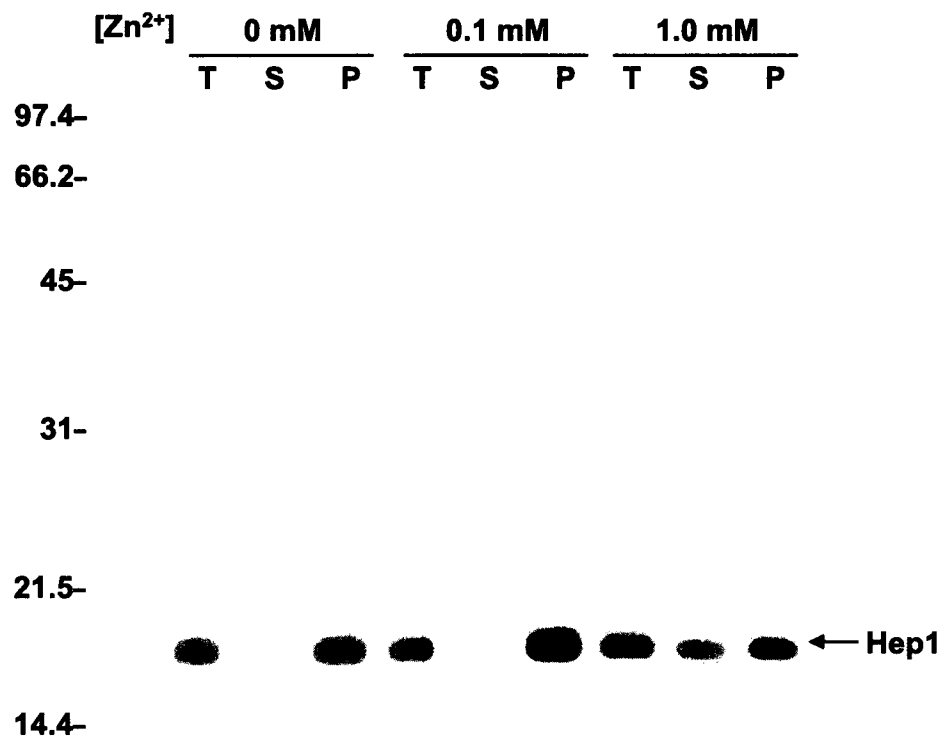


Figure 3.13. Effect of zinc on *S. cerevisiae* Hep1 solubility. SDS-PAGE analysis of *E. coli* overexpressing *S. cerevisiae* Hep1 in minimum medium (M9) containing the indicated concentrations of zinc chloride. Molecular weight markers (Lane 1) and Rosetta 2 *E. coli* harboring a plasmid for expressing *S. cerevisiae* Hep1 (A, lane 2-10). For each sample, 20 μ g of total protein from whole cells (T) is shown as well as the soluble lysates (S) and pellets (P) derived from a sample that contained the same amount of protein before fractionation.

examined the effect of Zn^{2+} removal on the solubility of purified yeast Hep1. MMTS and the zinc chelator EDTA were incubated with Hep1 in solutions of varying pH (7.0 to 8.0). Because MMTS displaces zinc ligands by converting free sulfhydryls on cysteines to dithiomethane moieties (Smith et al., 1975), DTT was added to reactions two minutes after MMTS to reduce the sulfhydryl groups on all four cysteines. Hep1 solubility was measured by monitoring increases in absorbance at 340 nm due to changes in turbidity. Figure 3.14 shows the effect of zinc removal on Hep1 solubility. Hep1 aggregated at 23 °C upon zinc removal under all conditions analyzed (pH = 7.0 to 8.0). However, the kinetics of protein aggregation were fastest at pH 8.0, as was the extent of aggregation over the time course of the assay. In the absence of MMTS treatment, Hep1 remained soluble for ≥ 48 hours at 23°C in 50 mM Tris pH 8.0 containing 0.5 mM EDTA.

3.3.10. Zn^{2+} is not required for the solubility of human Hep.

As described above, purified human Hep contains near stoichiometric zinc, suggesting that Zn^{+2} may be required for solubility. To test this, *E. coli* transformed with pHisHep were grown in minimum media (M9) containing 0 to 1 mM ZnCl_2 . Analysis of the soluble and insoluble cell fractions on SDS-PAGE revealed that soluble Hep is detectable within the soluble fraction when expressed in the absence of Zn^{2+} (Figure 3.15). The fraction of soluble Hep was greatest in cells grown in medium containing 0.1 mM Zn^{2+} , and the fraction of Hep that was soluble decreased when expression experiments were performed in medium containing higher concentrations of Zn^{2+} . I also investigated whether addition of MMTS to purified recombinant Hep affected its solubility like yeast Hep1. These experiments revealed that addition of MMTS to human Hep had not effect

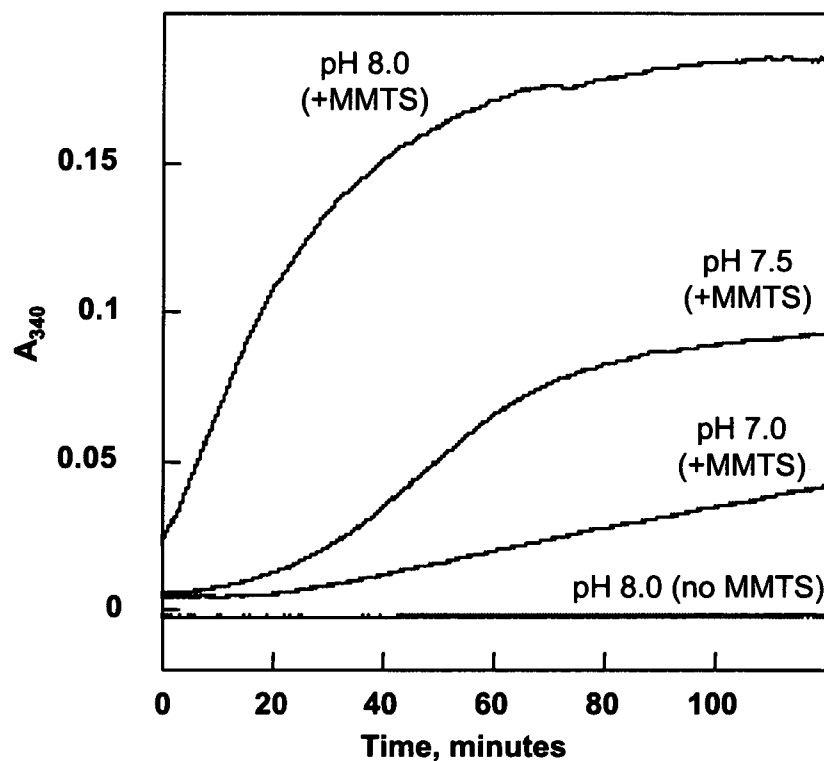


Figure 3.14. Zn^{2+} displacement leads to aggregation of *S. cerevisiae* Hep1. Effect of zinc removal on yeast Hep1 solubility. Reactions containing 10 μM Hep1, 1 mM EDTA, 50 mM buffer (HEPES pH 7.0, HEPES pH 7.5, or Tris pH 8.0) were incubated for 5 minutes at 23 °C. MMTS was added to a final concentration of 200 μM , reactions were incubated for 2 minutes, DTT was added to a final concentration of 1 mM, and absorbance changes were monitored at 340 nm at 23 °C. Similar reactions were performed in the absence of MMTS to evaluate the effects zinc removal.

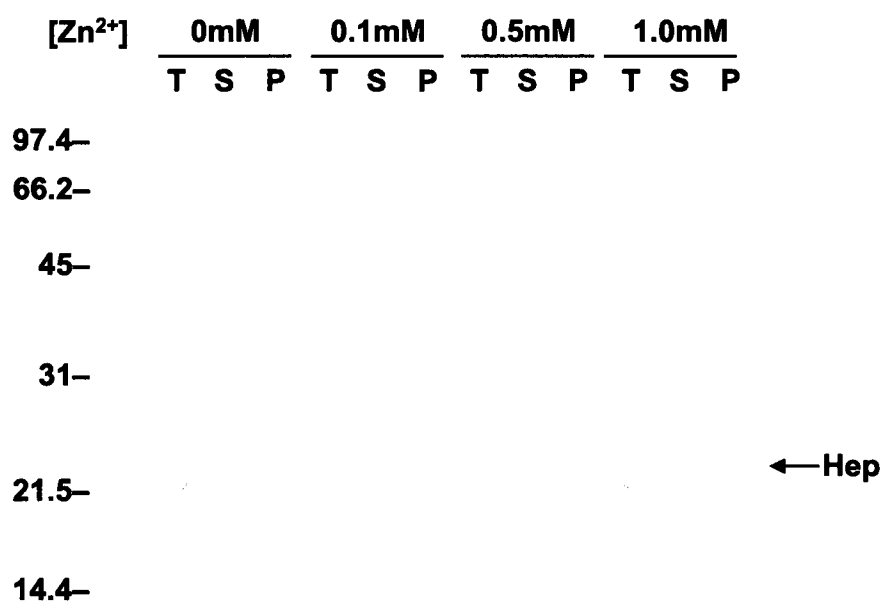


Figure 3.15. Effect of zinc on human Hep solubility. SDS-PAGE analysis of *E. coli* overexpressing human Hep in minimum medium (M9) containing the indicated concentrations of zinc chloride. Molecular weight markers (Lane 1) and Rosetta 2 *E. coli* harboring a plasmid for expressing human Hep (lane 2-13). For each sample, 20 µg of total protein from whole cells (T) is shown as well as the soluble lysates (S) and pellets (P) derived from a sample that contained the same amount of protein before fractionation.

on protein solubility when experiments were performed using 10 μM Hep. This finding suggests that Zn^{2+} may not be essential for maintaining the solubility of human Hep.

3.4. Discussion

In Chapter 2, I found that human Hep regulates mtHsp70 ATPase activity through an interaction with its N-terminal ATPase domain, and I showed that yeast Hep1 could interact with human mtHsp70, implicating a role for residues conserved among yeast and human escort proteins in mediating binding to their respective chaperones (Burri et al., 2004). However, the relative importance of these conserved residues in escort protein regulation of chaperone ATPase activity and solubility was not established. My results in this chapter provide evidence that yeast Hep1 can also stimulate the ATPase activity of mtHsp70, and they show that a histidine (His107) is required for human Hep stimulation of mtHsp70 ATPase activity that is conserved in other mitochondrial and chloroplast escort protein homologs (Willmund et al., 2008). Steady-state ATPase measurements found that Hep mutants having a H107A mutation alone and in combination with the non-disruptive W115I mutation did not elicit a detectable increase in the steady-state ATPase activity of 70^{ATPase} (or full-length mthsp70) when present at a concentration (40 μM) that is 10-fold higher than that required for half-maximal stimulation of 70^{ATPase} by Hep and Hep-W115I. This can be contrasted with the seven other alanine mutants characterized, which stimulated 70^{ATPase} activity up to 5-11 fold with half-maximal stimulation occurring at concentrations ranging from 6 to 38 μM .

I also present evidence in this chapter that Hep requires H107 to effectively bind mtHsp70 in the absence of nucleotides. Fluorescence measurements revealed that among

all the Hep mutants characterized the Hep-W115I/H107A requires the highest concentrations to quench 70^{ATPase} fluorescence. Whereas Hep-W115I decreased 70^{ATPase} fluorescence up to 26.6%, Hep-W115I/H107A only reduced chaperone fluorescence by 2.7% when present at a level that is 8-fold greater than that required for half-maximal quenching by Hep-W115I. These differences are interpreted as arising because alanine substitution of His107 decreases the binding affinity of Hep for 70^{ATPase} . Affinity purification and SPR measurements could not detect formation of a complex between Hep-W115I/H107A in the absence of nucleotide. Furthermore, SPR studies found that addition of ADP and ATP did not enhance Hep-W115I/H107A binding to 70^{ATPase} at a pH (8.0) corresponding to that of the mitochondrial matrix (Llopis et al., 1998). However, binding by this mutant was influenced by a small change in pH. SPR measurements performed at pH 7.4 detected greater binding of Hep-W115I/H107A to 70^{ATPase} in the presence of nucleotides. This finding can be contrasted with previous analysis of Hep and 70^{ATPase} binding, which found that nucleotides destabilize escort-chaperone protein complexes (Figure 2.8).

The results from yeast complementation experiments demonstrate that human Hep is a functional ortholog of yeast Hep1. As previously observed, a vector that constitutively expresses yeast Hep1 complemented the growth of Δhep1 *S. cerevisiae* (Momose et al., 2007; Yamamoto et al., 2005). Similarly, a vector designed to constitutively express human Hep complemented the growth of Δhep1 yeast, as well as vectors expressing human Hep with alanine substitutions at residues that have mild (R81) and no (K88) effects on Hep function *in vitro*. In contrast, human Hep-H107A could not complement Δhep1 yeast under similar conditions. This suggests that Hep requires this conserved

histidine to effectively regulate both yeast and human hsp70 molecular chaperones. There is evidence that the homologous histidine in Hep1 is part of a basic patch that is required for escort protein function. A Hep1 mutant having Arg106 and His107 mutated to alanine are incapable of supporting $\Delta hep1$ growth on a fermentable carbon source and ineffective at increasing the solubility of Ssc1 upon overexpression in *E. coli* (Momose et al., 2007). The solution structure of yeast Hep1 indicates that H107 is proximal to tetracysteine-bound zinc (Momose et al., 2007), which is also essential for function (Yamamoto et al., 2005).

Surprisingly, escort activity measurements performed within *E. coli* found that Hep-W115I/H107A and Hep-H107A are both capable of promoting the solubility of full-length mtHsp70. In contrast, *in vitro* studies with Hep-W115I/H107A revealed that this variant has severe defects in binding and regulating the ATPase domain of mtHsp70, and yeast complementation studies found that Hep-H107A cannot support $\Delta hep1$ yeast growth. These seemingly conflicting results are interpreted as arising because the activities of the Hep-H107A mutants are sensitive to small changes in pH. The H107A mutation exhibits the most severe defects in binding to 70^{ATPase} under conditions that reflect the pH (8.0) of the mitochondrial matrix (Llopis et al., 1998). However, SPR measurements indicate that these binding defects are reduced under conditions (pH 7.4) that more closely resemble the *E. coli* cytosol (Wilks and Slonczewski, 2007) where Hep-H107A (and Hep-W115I/H107A) are capable of promoting the solubility of full-length mtHsp70. The finding that Hep-W115I/H107A binds 70^{ATPase} to differing extents at pH 7.4 in the presence and absence of nucleotide further suggests that the mechanism of Hep binding to the different conformational states of mtHsp70 may differ.

Additional studies will be required to establish the cellular roles of mitochondrial and plastid escort proteins and to determine how they recognize their cognate molecular chaperones to regulate solubility and ATPase activity. A recent study reported that the minimal Hep1 binding motif in yeast Ssc1 includes the isolated ATPase domain and the polypeptide linker that connects this domain to the C-terminal substrate-binding domain (Blamowska, 2010). Whether this linker is also required for human Hep binding to mtHsp70 is not known, since all studies described in thesis use 70^{ATPase} variants that include this polypeptide. However, the finding that human Hep binds to ATP-bound 70^{ATPase} suggests that other residues within mtHsp70 may be important for binding to their cognate escort proteins.

Chapter 4

Conclusions

4.1. Escort proteins stimulate Hsp70 ATPase activity.

Prior to my studies on human Hep, the only known protein function for Hep1 family members was chaperone activity (Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Willmund et al., 2008). Genetic studies showed that yeast Hep1 is required for normal chaperone function, as loss of Hep1 causes phenotypes similar to those observed upon loss of the chaperones Ssc1 and Ssq1 (Kawai et al., 2001; Neupert and Brunner, 2002; Vickery and Cupp-Vickery, 2007), including compromised mitochondrial protein import (Burri et al., 2004; Yamamoto et al., 2005), decreased Fe-S-cluster protein maturation (Sanjuan Szklarz et al., 2005; Sichting et al., 2005), and fragmented morphology of mitochondria (Sanjuan Szklarz et al., 2005). This phenotype was interpreted as arising because Hep1 depletion leads to the aggregation of Ssc1 and Ssq1 (Sanjuan Szklarz et al., 2005; Sichting et al., 2005). In this thesis, I have identified a novel ATPase stimulatory activity for escort proteins that was not uncovered through previous genetic studies. I show that human Hep and yeast Hep1 both stimulate the steady-state ATPase activity of human mtHsp70 (Figure 2.12, and 3.6). In addition, I provide evidence that this stimulation arises because of an interaction between these escort proteins and the N-terminal mtHsp70 ATPase domain (Figure 2.9). A study with yeast Hep1 and Ssc1 subsequent to my initial finding showed that yeast Hep1 also regulates Ssc1 through an interaction with the ATPase domain (Blamowska, 2010). The biological role of escort

protein stimulation of chaperone catalytic activity is not yet known. However, the finding that escort proteins regulate conversion of mtHsp70 between its high and low peptide affinity states suggests that stimulation of ATPase activity could influence chaperone and substrate interactions, as observed with other cochaperones (Blamowska, 2010; Karzai and McMacken, 1996; Laufen et al., 1999; Liberek et al., 1991). Additional studies will be needed to determine if Hep regulates mtHsp70 and peptide binding, or if Hep cooperates with other cochaperones in their regulation of mtHsp70 activities, such as J-type cochaperones (Karzai and McMacken, 1996; Laufen et al., 1999; Liberek et al., 1991) and GrpE-like exchange factors (Brehmer et al., 2001; Harrison et al., 1997).

Human Hep may stimulate the ATPase activity of mtHsp70 by enhancing the rate of ATP hydrolysis like J-type auxiliary cochaperones (Misselwitz et al., 1998; Silberg and Vickery, 2000). Alternatively, Hep could stimulate the rate of nucleotide exchange, similar to GrpE-type cochaperones (Liberek et al., 1991). My findings from steady-state experiments suggest that Hep could regulate both kinetic steps in the mtHsp70 ATPase cycle, as biphasic kinetics were observed in steady-state ATP measurements examining Hep effects on full-length mtHsp70 (Figure 2.12 and 2.13). The initial fast phase is predicted to represent one of the initial kinetic steps in the ATPase reaction cycle, *e.g.*, ATP binding, R→T conversion, or ATP hydrolysis (Figure 4.1). The magnitude of this burst phase is consistent with a single turnover by mtHsp70. The subsequent slow phase is predicted to represent an ATPase reaction cycle step that becomes rate limiting under steady-state conditions, such as ADP release (Figure 4.2). Experiments examining the effect of Hep on the individual reaction cycle steps under presteady-state conditions will be required to determine the exact mechanism(s) by which Hep regulates mtHsp70 and

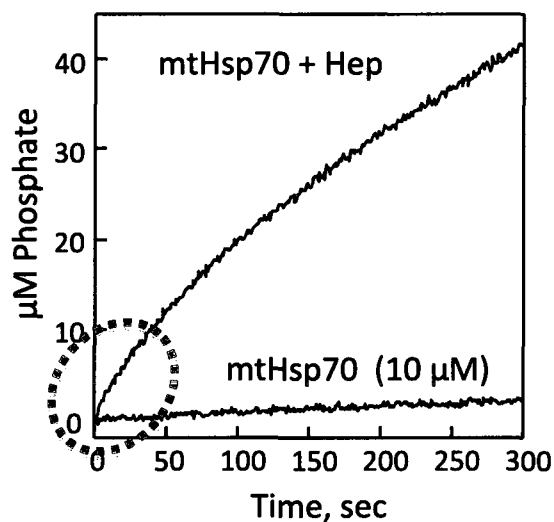
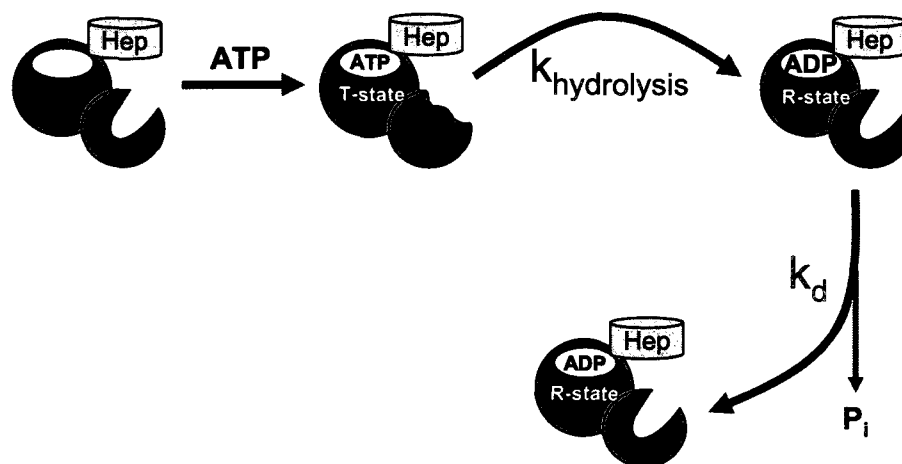


Figure 4.1. Possible mechanism for the initial fast phase of the biphasic kinetics in the ATPase assay of mtHsp70 and Hep. This phase may represent the single turn-over ATP hydrolysis. The strong stimulation (~49-fold) observed in this phase may be a direct result of the Hep stimulation on the ATP hydrolysis of mtHsp70.

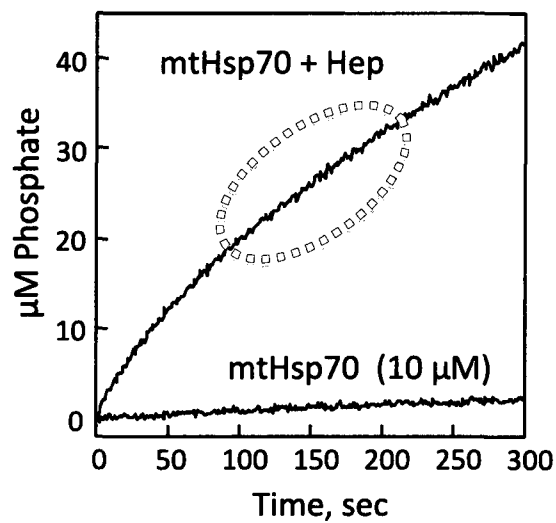
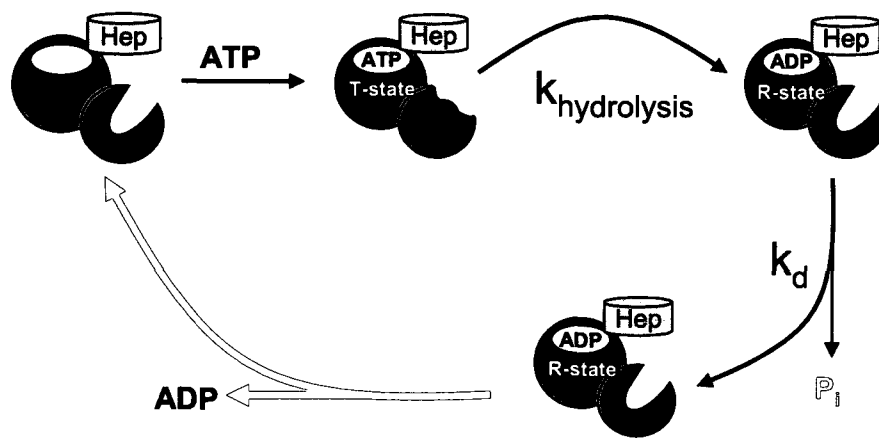


Figure 4.2. Possible mechanism for the slow phase of the biphasic kinetics in the ATPase assay of mtHsp70 and Hep. The consequent slow phase may represent the ATPase reaction cycle after the first round. In this step, the nucleotide exchange may be the rate limiting step. However, it remains unclear whether Hep also facilitate the nucleotide exchange like GrpE-type NEFs.

nucleotide interactions, as described for bacterial hsp70 family members (Russell et al., 1998; Silberg and Vickery, 2000). These experiments are only feasible now that I have discovered how to produce high levels of folded, functional mtHsp70 for *in vitro* biochemical studies, and identified conditions where mtHsp70 remains soluble in the absence of Hep (after purification).

4.2. Human Hep and yeast Hep1 are functional orthologs.

Prior to the work described herein, two escort protein homologs had been characterized, including yeast mitochondrial Hep1 (Burri et al., 2004; Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Yamamoto et al., 2005) and chlamydomonas plastid Hep2 (Willmund et al., 2008). Yeast genetic studies showed that mitochondrial escort proteins are capable of maintaining the chaperones Ssc1 and Ssq1 in soluble, functional conformations (Sanjuan Szklarz et al., 2005; Sichting et al., 2005). In addition, biochemical studies with the plastid escort protein from a single celled photosynthetic organism provided evidence that Hep2 is required for the *de novo* folding of its cognate Hsp70 (Willmund et al., 2008), like yeast Hep1 (Sichting et al., 2005). As shown in Figure 1.5, Hep homologs are also predicted to be within mitochondria and plastids of multicellular eukaryotes, such as coelenterates, worms, plants, and vertebrates. Although escort protein homologs were predicted to have conserved roles in these organelles, it was not known prior to this thesis if these proteins have similar functions as escort proteins found in unicellular fungi and algae or if they have additional activities.

My results provide multiple lines of evidence that mammalian escort proteins are functional orthologs of fungal escort proteins. First, analysis of human Hep localization

in tissue culture showed that human Hep is a mitochondria protein, like yeast Hep1 (Burri et al., 2004; Sanjuan Szklarz et al., 2005; Sichting et al., 2005). Second, escort activity analysis in *E. coli* revealed that human Hep and yeast Hep1 can both increase the levels of soluble mtHsp70 produced by *E. coli* upon overexpression from pET vectors (Figure 2.2 and 2.4). Third, *in vitro* studies showed that human Hep-mtHsp70 complexes are destabilized by ATP and ADP (Figure 2.7), like yeast Hep1-Ssc1 complexes (Sichting et al., 2005). Fourth, biochemical studies revealed that human Hep contains stoichiometric Zn^{+2} like yeast Hep1 (Momose et al., 2007). Fifth, human Hep was able to complement the growth defect of Δhep1 *S. cerevisiae* (Figure 3.11).

The results from my studies also suggest that human Hep may have functions not found in fungal escort proteins. I show that human Hep stimulates the steady-state ATPase activity of human mtHsp70 through an interaction with the ATPase domain (Figure 2.9 and 2.12). In addition, I demonstrate that yeast Hep1 stimulates the activity of the isolated mtHsp70 ATPase domain (Figure 3.3). In contrast, previous studies characterizing the effect of yeast Hep1 on Ssc1 catalytic activity had not observed ATPase stimulation (Sichting et al., 2005). It is possible that experiments performed to date have not detected effects of yeast Hep1 on Ssc1 and nucleotide interactions, even though yeast Hep1 alters the kinetics of ATP hydrolysis by Ssc1. All measurements reported have been performed under *steady-state* conditions, which would only observe a stimulatory effect if Hep1 accelerated the rate-limiting step in the overall Ssc1 ATPase reaction cycle. If Hep1 increases the rate of a step that is not rate-limiting for Ssc1 under steady-state conditions, then it would be challenging to detect its effect on that kinetic step. As noted above for mtHsp70, future studies will be needed to establish the kinetics

of the individual reaction steps of the Ssc1 ATPase reaction cycles (*e.g.*, $k_{\text{ATP binding}}$, $k_{\text{R} \rightarrow \text{T}}$ state, $k_{\text{ATP hydrolysis}}$, and $k_{\text{ADP dissociation}}$) and to determine how yeast Hep1 affects the rates of the individual reaction steps.

4.3. Mechanism of human Hep and mtHsp70 binding.

Previous studies have revealed that mitochondrial and plastid escort protein homologs form stable complexes with their cognate Hsp70 chaperones (Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Willmund et al., 2008). However, little is known about the structural details of these interactions. In my studies, I found that yeast Hep1 could interact with human mtHsp70 and stimulate its ATPase activity, despite their low sequence identity (<25%). This finding implicated a role for residues conserved among yeast and human escort proteins in mediating binding to their respective cognate chaperones. An alanine scan mutagenesis of conserved residues identified a histidine (His107) in human Hep that is required for the stimulation of ATPase activity and interaction of 70^{ATPase} at the resting pH (8.0) of mitochondria (Llopis et al., 1998). This residue is conserved in other mitochondrial and plastid escort protein homologs (Willmund et al., 2008). Furthermore, yeast complementation studies found that Hep-H107A cannot support Δhep1 yeast growth under conditions where both yeast Hep1 and human Hep complement growth (Figure 3.11). Surprisingly, escort activity measurements performed within *E. coli* found that H107A mutants retain the ability to promote the solubility of full-length mtHsp70. SPR studies verified that mtHsp70 binds more strongly to Hep-H107A under conditions that more closely reflect the conditions (pH = 7.4) within *E. coli* (Figure 3.9). Currently the mechanism that underlies this observation is not known. The close proximity of H107

and Zn^{+2} in the NMR structure of the core domain of Hep1 suggests that this histidine could participate as a ligand for zinc at pH 8.0 (Momose et al., 2007). In the absence of H107, a Zn bound conformation of Hep could be stabilized that is non-functional at pH 8.0, *e.g.*, tetracysteine-bound zinc. As pH is decreased to a level that is below the pKa for cysteines (≈ 8.3), the number of cysteines that coordinate the zinc could decrease as well, allowing for a Hep conformation that binds more effectively to human mtHsp70. Future mutagenesis studies examining the function of a H107C mutant will be required to address this hypothesis

I also present evidence that a truncated human Hep (residues 64-160) that is homologous to the trypsin-resistant core-domain of yeast Hep1 is sufficient to enhance the solubility of mtHsp70 *in vitro* and to stimulate the ATPase activity of 70^{ATPase}. In addition, characterization of smaller Hep truncation mutants (Figure 3.12) identified a new minimal region of Hep (residues 64-122) that is capable of increasing human mtHsp70 solubility upon expression in *E. coli*. Figure 4.3 shows the fragment of yeast Hep1 that is homologous to the fragment identified. This minimal motif, which corresponds to the most conserved region of escort proteins (Figure 1.5), contains both the tetracysteine motif and H107 that I identified as essential for Hep regulation of chaperone ATPase activity. Further experiments will be needed to determine if this 59 residue fragment is sufficient to stimulate the ATPase activity of mtHsp70 and complement the growth of Δhep1 yeast.

Although I provide evidence that Hep binds directly to the N-terminal ATPase domain of mtHsp70, the Hep binding region within mtHsp70 is not known. A recent study reported that a portion of the linker that connects ATPase domain to the C-terminal

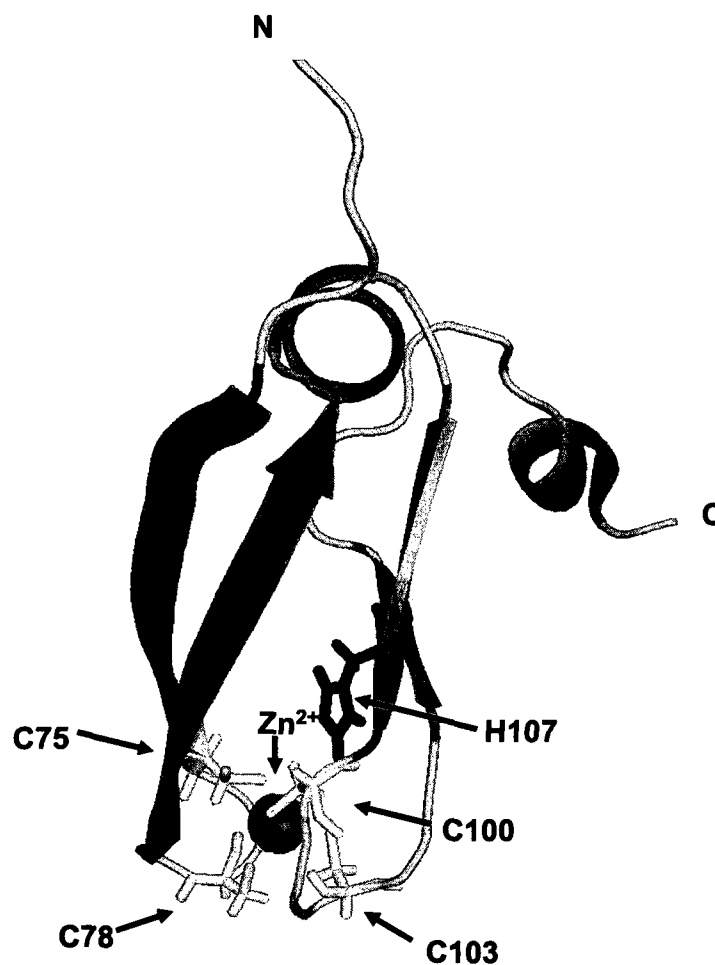


Figure 4.3. NMR structure of the region of *S. cerevisiae* Hep1 (PDB ID = 2E2Z) corresponding to the defined smallest function subdomain of human Hep (64-122). The four conserved cysteines, histidine 107 and bound Zn^{2+} ion are labeled. In the structure, H107 is proximal to the C100 and C103 in the tetracysteine motif.

substrate-binding domain in yeast Ssc1 (residues 412-415, “LVVV”) is crucial for the interaction to its cognate escort protein (Blamowska, 2010). The linker may also be important for human Hep regulation of mtHsp70, as residues from this linker are present in the 70^{ATPase} fragment used for the studies described herein. However, it is possible that this region is simply important for maintaining the ATPase domain in a conformation that binds strongly to escort proteins. Defining the Hep binding region within the mtHsp70 ATPase domain is expected to be challenging. Interaction of mtHsp70 to Hep is required for expressing soluble mtHsp70 *in vitro*, making it hard to screen for mutations in mtHsp70 that disrupt binding to Hep. Mutations that disrupt binding will also disrupt mtHsp70 folding, similar to that observed with mutations that simply destabilize the ATPase domain structure. Structural studies would aid in establishing how the core domain that I discovered interacts with the 70^{ATPase} domain. In addition, cross-linking studies could be used to identify Hep and 70^{ATPase} domain residues that are proximal, and the residues cross-linked could be mapped using mass spectrometry. One avenue to accomplish this would be to incorporate methionines into the core domain of Hep, which lacks methionines, and to use photomethionine incorporation to create mutants where the cross-linking is driven by a non-canonical amino acid placed in different locations within Hep (Suchanek et al., 2005).

4.4 Zinc regulation of escort proteins.

In my thesis research, I found that human Hep contains stoichiometric levels of bound Zn²⁺, similar to that observed with *S. cerevisiae* Hep1 (Momose et al., 2007). Recombinant *S. cerevisiae* Hep1 is only produced as a soluble protein in *E. coli* when

Zn^{2+} is added to the growth media (Figure 3.13). In addition, zinc-displacement by MMTS causes aggregation of purified yeast Hep1 over a range of pH values (7.0 to 8.0) (Figure 3.14). As shown in Chapter 3, human Hep also contains near stoichiometric level of zinc. However, it seems that Zn^{2+} is not essential for the solubility of human Hep. As shown in Figure 3.15, a small fraction of human Hep presents in the soluble fraction when expressed in the media without Zn^{2+} and the low concentration (0.1mM) of Zn^{2+} is optimal for Hep solubility. These results suggest Zn^{2+} is not an essential cofactor for human Hep to maintain in a soluble conformation. The role of Zn^{2+} on the function of human Hep has not yet been established. It is possible that human Hep requires Zn^{2+} to stay in a functional conformation to interact with its cognate chaperone. This hypothesis could be addressed by studying the interaction and ATPase activity of Zn^{2+} -free Hep and mtHsp70.

4.5 Human Hep regulation of mtHsp70 as a therapeutic target

Genetic and biochemical studies on escort proteins in unicellular organisms have shown that Hep family members are essential for the functions of their cognate chaperones (Sanjuan Szklarz et al., 2005; Sichting et al., 2005; Willmund et al., 2008). My data showing that human Hep is a functional ortholog of yeast Hep1 suggests that Hep will be needed to maintain human mtHsp70 in a folded and functional state in human cells. RNA interference studies will be required to determine if depletion of human *HEP* causes phenotypes consistent with loss of mitochondrial and cytosolic chaperone functions. In mitochondria, loss of Hep (and mtHsp70) activity is predicted to cause defects in mitochondrial protein folding, protein translocation, and the maturation

of Fe-S-cluster proteins (Bhattacharyya et al., 1995; Kaul et al., 2002). In the cytosol, loss of Hep (and mtHsp70) could also affect centrosome duplication (Ma et al., 2006). Increased levels of cytosolic mtHsp70 are correlated with the malignancy of cancers (Czarnecka et al., 2006; Wadhwa et al., 2006; Yi et al., 2008), and a direct interaction between mtHsp70 and p53 has been proposed to underlie this correlation (Ma et al., 2006; Wadhwa et al., 1998). If Hep is required for mtHsp70 to function in the cytosol, then the interaction between these two proteins would represent a possible target for the development of future cancer therapies (Bottger et al., 2008; Kaul et al., 2005).

Bibliography

- Anfinsen, C. B. (1973). Principles that govern the folding of protein chains. *Science* *181*, 223-230.
- Anfinsen, C. B., Harrington, W. F., Hvidt, A., Linderstrom-Lang, K., Ottesen, M., and Schellman, J. (1955). Studies on the structural basis of ribonuclease activity. *Biochim Biophys Acta* *17*, 141-142.
- Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., *et al.* (2004). The Pfam protein families database. *Nucleic Acids Res* *32*, D138-141.
- Bauer, M. F., Gempel, K., Reichert, A. S., Rappold, G. A., Lichtner, P., Gerbitz, K. D., Neupert, W., Brunner, M., and Hofmann, S. (1999). Genetic and structural characterization of the human mitochondrial inner membrane translocase. *J Mol Biol* *289*, 69-82.
- Baumann, F., Milisav, I., Neupert, W., and Herrmann, J. M. (2000). Ecm10, a novel hsp70 homolog in the mitochondrial matrix of the yeast *Saccharomyces cerevisiae*. *FEBS Lett* *487*, 307-312.
- Beinert, H., Holm, R. H., and Munck, E. (1997). Iron-sulfur clusters: nature's modular, multipurpose structures. *Science* *277*, 653-659.
- Ben-Zvi, A. P., and Goloubinoff, P. (2001). Review: mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones. *J Struct Biol* *135*, 84-93.
- Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* *3*, 1301-1306.
- Bhattacharyya, T., Karnezis, A. N., Murphy, S. P., Hoang, T., Freeman, B. C., Phillips, B., and Morimoto, R. I. (1995). Cloning and subcellular localization of human mitochondrial hsp70. *J Biol Chem* *270*, 1705-1710.
- Bitto, E., Bingman, C. A., Bittova, L., Kondrashov, D. A., Bannen, R. M., Fox, B. G., Markley, J. L., and Phillips, G. N., Jr. (2008). Structure of human J-type co-chaperone HscB reveals a tetracysteine metal-binding domain. *J Biol Chem* *283*, 30184-30192.
- Blamowska, M., Sichting M., Mapa K., (2010). ATPase domain and interdomain linker play a key role in aggregation of mitochondria Hsp70 chaperone Ssc1. *J Biol Chem* *in press*.

- Bonomi, F., Iametti, S., Morleo, A., Ta, D., and Vickery, L. E. (2008). Studies on the mechanism of catalysis of iron-sulfur cluster transfer from IscU[2Fe2S] by HscA/HscB chaperones. *Biochemistry* 47, 12795-12801.
- Borges, J. C., Fischer, H., Craievich, A. F., Hansen, L. D., and Ramos, C. H. (2003). Free human mitochondrial GrpE is a symmetric dimer in solution. *J Biol Chem* 278, 35337-35344.
- Bottger, S., Jerszyk, E., Low, B., and Walker, C. (2008). Genotoxic stress-induced expression of p53 and apoptosis in leukemic clam hemocytes with cytoplasmically sequestered p53. *Cancer Res* 68, 777-782.
- Brehmer, D., Rudiger, S., Gassler, C. S., Klostermeier, D., Packschies, L., Reinstein, J., Mayer, M. P., and Bukau, B. (2001). Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. *Nat Struct Biol* 8, 427-432.
- Brocchieri, L., Conway de Macario, E., and Macario, A. J. (2008). hsp70 genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evol Biol* 8, 19.
- Buchberger, A., Theyssen, H., Schroder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J., and Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J Biol Chem* 270, 16903-16910.
- Bukau, B., Deuerling, E., Pfund, C., and Craig, E. A. (2000). Getting newly synthesized proteins into shape. *Cell* 101, 119-122.
- Bukau, B., Weissman, J., and Horwich, A. (2006). Molecular chaperones and protein quality control. *Cell* 125, 443-451.
- Burkholder, W. F., Zhao, X., Zhu, X., Hendrickson, W. A., Gragerov, A., and Gottesman, M. E. (1996). Mutations in the C-terminal fragment of DnaK affecting peptide binding. *Proc Natl Acad Sci U S A* 93, 10632-10637.
- Burri, L., Vascotto, K., Fredersdorf, S., Tiedt, R., Hall, M. N., and Lithgow, T. (2004). Zim17, a novel zinc finger protein essential for protein import into mitochondria. *J Biol Chem* 279, 50243-50249.
- Cagney, G., Amiri, S., Premawaradena, T., Lindo, M., and Emili, A. (2003). In silico proteome analysis to facilitate proteomics experiments using mass spectrometry. *Proteome Sci* 1, 5.
- Chandramouli, K., and Johnson, M. K. (2006). HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. *Biochemistry* 45, 11087-11095.

- Chaudhuri, T. K., and Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. *Febs J* 273, 1331-1349.
- Claros, M. G., and Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* 241, 779-786.
- Craig, E. A., Kramer, J., and Kasic-Smithers, J. (1987). SSC1, a member of the 70-kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. *Proc Natl Acad Sci U S A* 84, 4156-4160.
- Craig, E. A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kasic-Smithers, J., and Nicolet, C. M. (1989). SSC1, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. *Mol Cell Biol* 9, 3000-3008.
- Craven, S. E., French, D., Ye, W., de Sauvage, F., and Rosenthal, A. (2005). Loss of Hspa9b in zebrafish recapitulates the ineffective hematopoiesis of the myelodysplastic syndrome. *Blood* 105, 3528-3534.
- Cupp-Vickery, J. R., Peterson, J. C., Ta, D. T., and Vickery, L. E. (2004). Crystal structure of the molecular chaperone HscA substrate binding domain complexed with the IscU recognition peptide ELPPVKIHC. *J Mol Biol* 342, 1265-1278.
- Czarnecka, A. M., Campanella, C., Zummo, G., and Cappello, F. (2006). Mitochondrial chaperones in cancer: from molecular biology to clinical diagnostics. *Cancer Biol Ther* 5, 714-720.
- D'Silva, P. D., Schilke, B., Walter, W., Andrew, A., and Craig, E. A. (2003). J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc Natl Acad Sci U S A* 100, 13839-13844.
- Daugaard, M., Rohde, M., and Jaattela, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett* 581, 3702-3710.
- Deloche, O., Kelley, W. L., and Georgopoulos, C. (1997). Structure-function analyses of the Ssc1p, Mdj1p, and Mge1p *Saccharomyces cerevisiae* mitochondrial proteins in *Escherichia coli*. *J Bacteriol* 179, 6066-6075.
- Deocaris, C. C., Kaul, S. C., and Wadhwa, R. (2006). On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60. *Cell Stress Chaperones* 11, 116-128.
- Deuerling, E., and Bukau, B. (2004). Chaperone-assisted folding of newly synthesized proteins in the cytosol. *Crit Rev Biochem Mol Biol* 39, 261-277.
- Dobson, C. M., and Karplus, M. (1999). The fundamentals of protein folding: bringing together theory and experiment. *Curr Opin Struct Biol* 9, 92-101.

- Dutkiewicz, R., Marszalek, J., Schilke, B., Craig, E. A., Lill, R., and Muhlenhoff, U. (2006). The Hsp70 chaperone Ssq1p is dispensable for iron-sulfur cluster formation on the scaffold protein Isu1p. *J Biol Chem* 281, 7801-7808.
- Dutkiewicz, R., Schilke, B., Cheng, S., Knieszner, H., Craig, E. A., and Marszalek, J. (2004). Sequence-specific interaction between mitochondrial Fe-S scaffold protein Isu and Hsp70 Ssq1 is essential for their in vivo function. *J Biol Chem* 279, 29167-29174.
- Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., and Marszalek, J. (2003). Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis. Similarities to and differences from its bacterial counterpart. *J Biol Chem* 278, 29719-29727.
- Ellis, R. J. (2001). Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci* 26, 597-604.
- Elsner, S., Simian, D., Iosefson, O., Marom, M., and Azem, A. (2009). The Mitochondrial Protein Translocation Motor: Structural Conservation between the Human and Yeast Tim14/Pam18-Tim16/Pam16 co-Chaperones. *Int J Mol Sci* 10, 2041-2053.
- Flaherty, K. M., DeLuca-Flaherty, C., and McKay, D. B. (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* 346, 623-628.
- Frydman, J. (2001). Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu Rev Biochem* 70, 603-647.
- Gaestel, M. (2006). Molecular chaperones in signal transduction. *Handb Exp Pharmacol*, 93-109.
- Gambill, B. D., Voos, W., Kang, P. J., Miao, B., Langer, T., Craig, E. A., and Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J Cell Biol* 123, 109-117.
- Garrido, C., Schmitt, E., Cande, C., Vahsen, N., Parcellier, A., and Kroemer, G. (2003). HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. *Cell Cycle* 2, 579-584.
- Genevaux, P., Georgopoulos, C., and Kelley, W. L. (2007). The Hsp70 chaperone machines of *Escherichia coli*: a paradigm for the repartition of chaperone functions. *Mol Microbiol* 66, 840-857.
- Gerner, E. W., Boone, R., Connor, W. G., Hicks, J. A., and Boone, M. L. (1976). A transient thermotolerant survival response produced by single thermal doses in HeLa cells. *Cancer Res* 36, 1035-1040.
- Gill, S. C., and von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182, 319-326.

- Glick, B. S. (1995). Can Hsp70 proteins act as force-generating motors? *Cell* 80, 11-14.
- Glick, B. S., Wachter, C., Reid, G. A., and Schatz, G. (1993). Import of cytochrome b2 to the mitochondrial intermembrane space: the tightly folded heme-binding domain makes import dependent upon matrix ATP. *Protein Sci* 2, 1901-1917.
- Gottesman, M. E., and Hendrickson, W. A. (2000). Protein folding and unfolding by *Escherichia coli* chaperones and chaperonins. *Curr Opin Microbiol* 3, 197-202.
- Greene, M. K., Maskos, K., and Landry, S. J. (1998). Role of the J-domain in the cooperation of Hsp40 with Hsp70. *Proc Natl Acad Sci U S A* 95, 6108-6113.
- Harrison, C. J., Hayer-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. (1997). Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* 276, 431-435.
- Hartl, F. U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852-1858.
- Helmbrecht, K., Zeise, E., and Rensing, L. (2000). Chaperones in cell cycle regulation and mitogenic signal transduction: a review. *Cell Prolif* 33, 341-365.
- Hendrick, J. P., and Hartl, F. U. (1995). The role of molecular chaperones in protein folding. *Faseb J* 9, 1559-1569.
- Henle, K. J., Karamuz, J. E., and Leeper, D. B. (1978). Induction of thermotolerance in Chinese hamster ovary cells by high (45 degrees) or low (40 degrees) hyperthermia. *Cancer Res* 38, 570-574.
- Herrmann, J. M., and Neupert, W. (2000). Protein transport into mitochondria. *Curr Opin Microbiol* 3, 210-214.
- Herrmann, J. M., Stuart, R. A., Craig, E. A., and Neupert, W. (1994). Mitochondrial heat shock protein 70, a molecular chaperone for proteins encoded by mitochondrial DNA. *J Cell Biol* 127, 893-902.
- Hoff, K. G., Silberg, J. J., and Vickery, L. E. (2000). Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. *Proc Natl Acad Sci U S A* 97, 7790-7795.
- Hoff, K. G., Ta, D. T., Tapley, T. L., Silberg, J. J., and Vickery, L. E. (2002). Hsc66 substrate specificity is directed toward a discrete region of the iron-sulfur cluster template protein IscU. *J Biol Chem* 277, 27353-27359.
- Hohfeld, J., Cyr, D. M., and Patterson, C. (2001). From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep* 2, 885-890.

- Horrigan, S. K., Arbieva, Z. H., Xie, H. Y., Kravarusic, J., Fulton, N. C., Naik, H., Le, T. T., and Westbrook, C. A. (2000). Delineation of a minimal interval and identification of 9 candidates for a tumor suppressor gene in malignant myeloid disorders on 5q31. *Blood* 95, 2372-2377.
- Horst, M., Oppliger, W., Rospert, S., Schonfeld, H. J., Schatz, G., and Azem, A. (1997). Sequential action of two hsp70 complexes during protein import into mitochondria. *Embo J* 16, 1842-1849.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61-68.
- Hunt, J. B., Neece, S. H., and Ginsburg, A. (1985). The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from *Escherichia coli* aspartate transcarbamoylase. *Anal Biochem* 146, 150-157.
- Jaattela, M., Saksela, K., and Saksela, E. (1989). Heat shock protects WEHI-164 target cells from the cytotoxicity by tumor necrosis factors alpha and beta. *Eur J Immunol* 19, 1413-1417.
- Jin, J., Hulette, C., Wang, Y., Zhang, T., Pan, C., Wadhwa, R., and Zhang, J. (2006). Proteomic identification of a stress protein, mortalin/mthsp70/GRP75: relevance to Parkinson disease. *Mol Cell Proteomics* 5, 1193-1204.
- Johnston, R. N., and Kucey, B. L. (1988). Competitive inhibition of hsp70 gene expression causes thermosensitivity. *Science* 242, 1551-1554.
- Kanai, M., Ma, Z., Izumi, H., Kim, S. H., Mattison, C. P., Winey, M., and Fukasawa, K. (2007). Physical and functional interaction between mortalin and Mps1 kinase. *Genes Cells* 12, 797-810.
- Kang, P. J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* 348, 137-143.
- Karzai, A. W., and McMacken, R. (1996). A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. *J Biol Chem* 271, 11236-11246.
- Kaul, S. C., Aida, S., Yaguchi, T., Kaur, K., and Wadhwa, R. (2005). Activation of wild type p53 function by its mortalin-binding, cytoplasmically localizing carboxyl terminus peptides. *J Biol Chem* 280, 39373-39379.
- Kaul, S. C., Deocaris, C. C., and Wadhwa, R. (2007). Three faces of mortalin: a housekeeper, guardian and killer. *Exp Gerontol* 42, 263-274.

Kaul, S. C., Reddel, R. R., Mitsui, Y., and Wadhwa, R. (2001). An N-terminal region of mot-2 binds to p53 in vitro. *Neoplasia* 3, 110-114.

Kaul, S. C., Taira, K., Pereira-Smith, O. M., and Wadhwa, R. (2002). Mortalin: present and prospective. *Exp Gerontol* 37, 1157-1164.

Kaul, S. C., Yaguchi, T., Taira, K., Reddel, R. R., and Wadhwa, R. (2003). Overexpressed mortalin (mot-2)/mthsp70/GRP75 and hTERT cooperate to extend the in vitro lifespan of human fibroblasts. *Exp Cell Res* 286, 96-101.

Kaul, S. C., Reddel, R. R., Sugiharac, T., Mitsui, Y., and Wadhwa, R. (2000). Inactivation of p53 and life span extension of human diploid fibroblasts by mot-2. *FEBS Lett* 474, 159-164.

Kawai, A., Nishikawa, S., Hirata, A., and Endo, T. (2001). Loss of the mitochondrial Hsp70 functions causes aggregation of mitochondria in yeast cells. *J Cell Sci* 114, 3565-3574.

Kim, R., Saxena, S., Gordon, D. M., Pain, D., and Dancis, A. (2001). J-domain protein, Jac1p, of yeast mitochondria required for iron homeostasis and activity of Fe-S cluster proteins. *J Biol Chem* 276, 17524-17532.

Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L., *et al.* (2006). Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* 23, 607-618.

Kimura, K., Tanaka, N., Nakamura, N., Takano, S., and Ohkuma, S. (2007). Knockdown of mitochondrial heat shock protein 70 promotes progeria-like phenotypes in *caenorhabditis elegans*. *J Biol Chem* 282, 5910-5918.

Knight, S. A., Sepuri, N. B., Pain, D., and Dancis, A. (1998). Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J Biol Chem* 273, 18389-18393.

Kronidou, N. G., Oppliger, W., Bolliger, L., Hannavy, K., Glick, B. S., Schatz, G., and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc Natl Acad Sci U S A* 91, 12818-12822.

Kurzrock, R. (2002). Myelodysplastic syndrome overview. *Semin Hematol* 39, 18-25.

Lakowicz, J. R. (2006). *Principles of Fluorescence Spectroscopy*, 3rd edn (New York: Springer).

Laloraya, S., Dekker, P. J., Voos, W., Craig, E. A., and Pfanner, N. (1995). Mitochondrial GrpE modulates the function of matrix Hsp70 in translocation and maturation of preproteins. *Mol Cell Biol* 15, 7098-7105.

- Laloraya, S., Gambill, B. D., and Craig, E. A. (1994). A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. *Proc Natl Acad Sci U S A* 91, 6481-6485.
- Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999). Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc Natl Acad Sci U S A* 96, 5452-5457.
- Lelivelt, M. J., and Kawula, T. H. (1995). Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. *J Bacteriol* 177, 4900-4907.
- Li, G. C., Li, L. G., Liu, Y. K., Mak, J. Y., Chen, L. L., and Lee, W. M. (1991). Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. *Proc Natl Acad Sci U S A* 88, 1681-1685.
- Li, G. C., and Werb, Z. (1982). Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc Natl Acad Sci U S A* 79, 3218-3222.
- Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc Natl Acad Sci U S A* 88, 2874-2878.
- Liu, T. X., Zhou, Y., Kanki, J. P., Deng, M., Rhodes, J., Yang, H. W., Sheng, X. M., Zon, L. I., and Look, A. T. (2002). Evolutionary conservation of zebrafish linkage group 14 with frequently deleted regions of human chromosome 5 in myeloid malignancies. *Proc Natl Acad Sci U S A* 99, 6136-6141.
- Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad Sci U S A* 95, 6803-6808.
- Lutz, T., Westermann, B., Neupert, W., and Herrmann, J. M. (2001). The mitochondrial proteins Ssq1 and Jac1 are required for the assembly of iron sulfur clusters in mitochondria. *J Mol Biol* 307, 815-825.
- Ma, Z., Izumi, H., Kanai, M., Kabuyama, Y., Ahn, N. G., and Fukasawa, K. (2006). Mortalin controls centrosome duplication via modulating centrosomal localization of p53. *Oncogene* 25, 5377-5390.
- Mach, H., Middaugh, C. R., and Lewis, R. V. (1992). Statistical determination of the average values of the extinction coefficients of tryptophan and tyrosine in native proteins. *Anal Biochem* 200, 74-80.
- Mankin, A. S. (2006). Nascent peptide in the "birth canal" of the ribosome. *Trends Biochem Sci* 31, 11-13.

- Martinez-Yamout, M., Legge, G. B., Zhang, O., Wright, P. E., and Dyson, H. J. (2000). Solution structure of the cysteine-rich domain of the Escherichia coli chaperone protein DnaJ. *J Mol Biol* 300, 805-818.
- Matouschek, A., Pfanner, N., and Voos, W. (2000). Protein unfolding by mitochondria. The Hsp70 import motor. *EMBO Rep* 1, 404-410.
- Mayer, M. P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* 62, 670-684.
- Mayer, M. P., Schroder, H., Rudiger, S., Paal, K., Laufen, T., and Bukau, B. (2000). Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat Struct Biol* 7, 586-593.
- McCarty, J. S., Buchberger, A., Reinstein, J., and Bukau, B. (1995). The role of ATP in the functional cycle of the DnaK chaperone system. *J Mol Biol* 249, 126-137.
- Miao, B., Davis, J. E., and Craig, E. A. (1997). Mge1 functions as a nucleotide release factor for Ssc1, a mitochondrial Hsp70 of Saccharomyces cerevisiae. *J Mol Biol* 265, 541-552.
- Misselwitz, B., Staack, O., and Rapoport, T. A. (1998). J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. *Mol Cell* 2, 593-603.
- Momose, T., Ohshima, C., Maeda, M., and Endo, T. (2007). Structural basis of functional cooperation of Tim15/Zim17 with yeast mitochondrial Hsp70. *EMBO Rep* 8, 664-670.
- Moran, L., Mirault, M. E., Arrigo, A. P., Goldschmidt-Clermont, M., and Tissieres, A. (1978). Heat shock of Drosophila melanogaster induces the synthesis of new messenger RNAs and proteins. *Philos Trans R Soc Lond B Biol Sci* 283, 391-406.
- Morano, K. A. (2007). New tricks for an old dog: the evolving world of Hsp70. *Ann N Y Acad Sci* 1113, 1-14.
- Moro, F., Sirrenberg, C., Schneider, H. C., Neupert, W., and Brunner, M. (1999). The TIM17.23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix. *Embo J* 18, 3667-3675.
- Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003). Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *Embo J* 22, 4815-4825.
- Muhlenhoff, U., and Lill, R. (2000). Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. *Biochim Biophys Acta* 1459, 370-382.

- Muhlenhoff, U., Richhardt, N., Gerber, J., and Lill, R. (2002). Characterization of iron-sulfur protein assembly in isolated mitochondria. A requirement for ATP, NADH, and reduced iron. *J Biol Chem* 277, 29810-29816.
- Mumberg, D., Muller, R., and Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156, 119-122.
- Naylor, D. J., Stines, A. P., Hoogenraad, N. J., and Hoj, P. B. (1998). Evidence for the existence of distinct mammalian cytosolic, microsomal, and two mitochondrial GrpE-like proteins, the Co-chaperones of specific Hsp70 members. *J Biol Chem* 273, 21169-21177.
- Neupert, W., and Brunner, M. (2002). The protein import motor of mitochondria. *Nat Rev Mol Cell Biol* 3, 555-565.
- Neupert, W., Hartl, F. U., Craig, E. A., and Pfanner, N. (1990). How do polypeptides cross the mitochondrial membranes? *Cell* 63, 447-450.
- Nissen, P., Hansen, J., Ban, N., Moore, P. B., and Steitz, T. A. (2000). The structural basis of ribosome activity in peptide bond synthesis. *Science* 289, 920-930.
- Oliveira, C. L., Borges, J. C., Torriani, I. L., and Ramos, C. H. (2006). Low resolution structure and stability studies of human GrpE#2, a mitochondrial nucleotide exchange factor. *Arch Biochem Biophys* 449, 77-86.
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 4, 2411-2423.
- Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J., and Fink, A. L. (1993). ATP-induced protein-Hsp70 complex dissociation requires K⁺ but not ATP hydrolysis. *Nature* 365, 664-666.
- Pelham, H. R. (1984). Hsp70 accelerates the recovery of nucleolar morphology after heat shock. *Embo J* 3, 3095-3100.
- Pellecchia, M., Montgomery, D. L., Stevens, S. Y., Vander Kooi, C. W., Feng, H. P., Gierasch, L. M., and Zuiderweg, E. R. (2000). Structural insights into substrate binding by the molecular chaperone DnaK. *Nat Struct Biol* 7, 298-303.
- Petersen, N. S., and Mitchell, H. K. (1981). Recovery of protein synthesis after heat shock: prior heat treatment affects the ability of cells to translate mRNA. *Proc Natl Acad Sci U S A* 78, 1708-1711.
- Prip-Buus, C., Westerman, B., Schmitt, M., Langer, T., Neupert, W., and Schwarz, E. (1996). Role of the mitochondrial DnaJ homologue, Mdj1p, in the prevention of heat-induced protein aggregation. *FEBS Lett* 380, 142-146.
- Qiu, X. B., Shao, Y. M., Miao, S., and Wang, L. (2006). The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cell Mol Life Sci* 63, 2560-2570.

Rassow, J., Maarse, A. C., Krainer, E., Kubrich, M., Muller, H., Meijer, M., Craig, E. A., and Pfanner, N. (1994). Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J Cell Biol* 127, 1547-1556.

Reid, K. L., and Fink, A. L. (1996). Physical interactions between members of the DnaK chaperone machinery: characterization of the DnaK.GrpE complex. *Cell Stress Chaperones* 1, 127-137.

Revington, M., Zhang, Y., Yip, G. N., Kurochkin, A. V., and Zuiderweg, E. R. (2005). NMR investigations of allosteric processes in a two-domain *Thermus thermophilus* Hsp70 molecular chaperone. *J Mol Biol* 349, 163-183.

Riabowol, K. T., Mizzen, L. A., and Welch, W. J. (1988). Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. *Science* 242, 433-436.

Ritossa, F. (1962). A new puffing pattern induced by heat shock and DNP in *Drosophila*. *Experientia* 18, 571-573.

Rockmill, B., Lambie, E. J., and Roeder, G. S. (1991). Spore enrichment. *Methods Enzymol* 194, 146-149.

Rowley, N., Prip-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barrell, B., and Neupert, W. (1994). Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. *Cell* 77, 249-259.

Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *Embo J* 16, 1501-1507.

Russell, R., Jordan, R., and McMacken, R. (1998). Kinetic characterization of the ATPase cycle of the DnaK molecular chaperone. *Biochemistry* 37, 596-607.

Ryan, M. T., and Pfanner, N. (2001). Hsp70 proteins in protein translocation. *Adv Protein Chem* 59, 223-242.

Samanta, M. P., and Liang, S. (2003). Predicting protein functions from redundancies in large-scale protein interaction networks. *Proc Natl Acad Sci U S A* 100, 12579-12583.

Sanjuan Szklarz, L. K., Guiard, B., Rissler, M., Wiedemann, N., Kozjak, V., van der Laan, M., Lohaus, C., Marcus, K., Meyer, H. E., Chacinska, A., *et al.* (2005). Inactivation of the mitochondrial heat shock protein zim17 leads to aggregation of matrix hsp70s followed by pleiotropic effects on morphology and protein biogenesis. *J Mol Biol* 351, 206-218.

Sapareto, S. A., Hopwood, L. E., Dewey, W. C., Raju, M. R., and Gray, J. W. (1978). Effects of hyperthermia on survival and progression of Chinese hamster ovary cells. *Cancer Res* 38, 393-400.

Savel'ev, A. S., Novikova, L. A., Kovaleva, I. E., Luzikov, V. N., Neupert, W., and Langer, T. (1998). ATP-dependent proteolysis in mitochondria. m-AAA protease and PIM1 protease exert overlapping substrate specificities and cooperate with the mtHsp70 system. *J Biol Chem* 273, 20596-20602.

Schilke, B., Forster, J., Davis, J., James, P., Walter, W., Laloraya, S., Johnson, J., Miao, B., and Craig, E. (1996). The cold sensitivity of a mutant of *Saccharomyces cerevisiae* lacking a mitochondrial heat shock protein 70 is suppressed by loss of mitochondrial DNA. *J Cell Biol* 134, 603-613.

Schilke, B., Voisine, C., Beinert, H., and Craig, E. (1999). Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 96, 10206-10211.

Schilke, B., Williams, B., Knieszner, H., Puksza, S., D'Silva, P., Craig, E. A., and Marszalek, J. (2006). Evolution of mitochondrial chaperones utilized in Fe-S cluster biogenesis. *Curr Biol* 16, 1660-1665.

Schmid, D., Baici, A., Gehring, H., and Christen, P. (1994). Kinetics of molecular chaperone action. *Science* 263, 971-973.

Schneider, H. C., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* 371, 768-774.

Schneider, H. C., Westermann, B., Neupert, W., and Brunner, M. (1996). The nucleotide exchange factor MGE exerts a key function in the ATP-dependent cycle of mt-Hsp70-Tim44 interaction driving mitochondrial protein import. *Embo J* 15, 5796-5803.

Shi, M., Jin, J., Wang, Y., Beyer, R. P., Kitsou, E., Albin, R. L., Gearing, M., Pan, C., and Zhang, J. (2008). Mortalin: a protein associated with progression of Parkinson disease? *J Neuropathol Exp Neurol* 67, 117-124.

Sichting, M., Mokranjac, D., Azem, A., Neupert, W., and Hell, K. (2005). Maintenance of structure and function of mitochondrial Hsp70 chaperones requires the chaperone Hsp1. *Embo J* 24, 1046-1056.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H. E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., *et al.* (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci U S A* 100, 13207-13212.

Siegenthaler, R. K., and Christen, P. (2005). The importance of having thermosensor control in the DnaK chaperone system. *J Biol Chem* 280, 14395-14401.

Siegenthaler, R. K., and Christen, P. (2006). Tuning of DnaK chaperone action by nonnative protein sensor DnaJ and thermosensor GrpE. *J Biol Chem* 281, 34448-34456.

- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19-27.
- Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vickery, L. E. (2001). The Fe/S assembly protein IscU behaves as a substrate for the molecular chaperone Hsc66 from *Escherichia coli*. *J Biol Chem* 276, 1696-1700.
- Silberg, J. J., Hoff, K. G., and Vickery, L. E. (1998). The Hsc66-Hsc20 chaperone system in *Escherichia coli*: chaperone activity and interactions with the DnaK-DnaJ-grpE system. *J Bacteriol* 180, 6617-6624.
- Silberg, J. J., Tapley, T. L., Hoff, K. G., and Vickery, L. E. (2004). Regulation of the HscA ATPase reaction cycle by the co-chaperone HscB and the iron-sulfur cluster assembly protein IscU. *J Biol Chem* 279, 53924-53931.
- Silberg, J. J., and Vickery, L. E. (2000). Kinetic characterization of the ATPase cycle of the molecular chaperone Hsc66 from *Escherichia coli*. *J Biol Chem* 275, 7779-7786.
- Silver, I., and Erecinska, M. (1998). Oxygen and ion concentrations in normoxic and hypoxic brain cells. *Adv Exp Med Biol* 454, 7-16.
- Simon, S. M., and Blobel, G. (1993). Mechanisms of translocation of proteins across membranes. *Subcell Biochem* 21, 1-15.
- Simon, S. M., Peskin, C. S., and Oster, G. F. (1992). What drives the translocation of proteins? *Proc Natl Acad Sci U S A* 89, 3770-3774.
- Slepenkov, S. V., and Witt, S. N. (1998). Peptide-induced conformational changes in the molecular chaperone DnaK. *Biochemistry* 37, 16749-16756.
- Smith, D. J., Maggio, E. T., and Kenyon, G. L. (1975). Simple alkanethiol groups for temporary blocking of sulfhydryl groups of enzymes. *Biochemistry* 14, 766-771.
- Strain, J., Lorenz, C. R., Bode, J., Garland, S., Smolen, G. A., Ta, D. T., Vickery, L. E., and Culotta, V. C. (1998). Suppressors of superoxide dismutase (SOD1) deficiency in *Saccharomyces cerevisiae*. Identification of proteins predicted to mediate iron-sulfur cluster assembly. *J Biol Chem* 273, 31138-31144.
- Suchanek, M., Radzikowska, A., and Thiele, C. (2005). Photo-leucine and photo-methionine allow identification of protein-protein interactions in living cells. *Nat Methods* 2, 261-267.
- Sun, G., Gargus, J. J., Ta, D. T., and Vickery, L. E. (2003). Identification of a novel candidate gene in the iron-sulfur pathway implicated in ataxia-susceptibility: human gene encoding HscB, a J-type co-chaperone. *J Hum Genet* 48, 415-419.

- Swain, J. F., Dinler, G., Sivendran, R., Montgomery, D. L., Stotz, M., and Gierasch, L. M. (2007). Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker. *Mol Cell* 26, 27-39.
- Tachibana, T., Astumi, S., Shioda, R., Ueno, M., Uritani, M., and Ushimaru, T. (2002). A novel non-conventional heat shock element regulates expression of MDJ1 encoding a DnaJ homolog in *Saccharomyces cerevisiae*. *J Biol Chem* 277, 22140-22146.
- Takeda, S., and McKay, D. B. (1996). Kinetics of peptide binding to the bovine 70 kDa heat shock cognate protein, a molecular chaperone. *Biochemistry* 35, 4636-4644.
- Tang, Y. C., Chang, H. C., Roeben, A., Wischniewski, D., Wischniewski, N., Kerner, M. J., Hartl, F. U., and Hayer-Hartl, M. (2006). Structural features of the GroEL-GroES nanocage required for rapid folding of encapsulated protein. *Cell* 125, 903-914.
- Tissieres, A., Mitchell, H. K., and Tracy, U. M. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. *J Mol Biol* 85, 389-398.
- Tong, W. H., and Rouault, T. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. *Embo J* 19, 5692-5700.
- Trzesniewska, K., Brzyska, M., and Elbaum, D. (2004). Neurodegenerative aspects of protein aggregation. *Acta Neurobiol Exp (Wars)* 64, 41-52.
- Ungermann, C., Guiard, B., Neupert, W., and Cyr, D. M. (1996). The delta psi- and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria. *Embo J* 15, 735-744.
- Vergilio, J. A., and Bagg, A. (2003). Myelodysplastic syndromes. Contemporary biologic concepts and emerging diagnostic approaches. *Am J Clin Pathol* 119 Suppl, S58-77.
- Vickery, L. E., and Cupp-Vickery, J. R. (2007). Molecular chaperones HscA/Ssq1 and HscB/Jac1 and their roles in iron-sulfur protein maturation. *Crit Rev Biochem Mol Biol* 42, 95-111.
- Vickery, L. E., Silberg, J. J., and Ta, D. T. (1997). Hsc66 and Hsc20, a new heat shock cognate molecular chaperone system from *Escherichia coli*. *Protein Sci* 6, 1047-1056.
- Voisine, C., Cheng, Y. C., Ohlson, M., Schilke, B., Hoff, K., Beinert, H., Marszalek, J., and Craig, E. A. (2001). Jac1, a mitochondrial J-type chaperone, is involved in the biogenesis of Fe/S clusters in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 98, 1483-1488.
- Voisine, C., Schilke, B., Ohlson, M., Beinert, H., Marszalek, J., and Craig, E. A. (2000). Role of the mitochondrial Hsp70s, Ssc1 and Ssq1, in the maturation of Yfh1. *Mol Cell Biol* 20, 3677-3684.

Voos, W., Gambill, B. D., Laloraya, S., Ang, D., Craig, E. A., and Pfanner, N. (1994). Mitochondrial GrpE is present in a complex with hsp70 and preproteins in transit across membranes. *Mol Cell Biol* 14, 6627-6634.

Voos, W., and Rottgers, K. (2002). Molecular chaperones as essential mediators of mitochondrial biogenesis. *Biochim Biophys Acta* 1592, 51-62.

Wadhwa, R., Pereira-Smith, O. M., Reddel, R. R., Sugimoto, Y., Mitsui, Y., and Kaul, S. C. (1995). Correlation between complementation group for immortality and the cellular distribution of mortalin. *Exp Cell Res* 216, 101-106.

Wadhwa, R., Takano, S., Kaur, K., Deocaris, C. C., Pereira-Smith, O. M., Reddel, R. R., and Kaul, S. C. (2006). Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. *Int J Cancer* 118, 2973-2980.

Wadhwa, R., Takano, S., Robert, M., Yoshida, A., Nomura, H., Reddel, R. R., Mitsui, Y., and Kaul, S. C. (1998). Inactivation of tumor suppressor p53 by mot-2, a hsp70 family member. *J Biol Chem* 273, 29586-29591.

Wadhwa, R., Takano, S., Taira, K., and Kaul, S. C. (2004). Reduction in mortalin level by its antisense expression causes senescence-like growth arrest in human immortalized cells. *J Gene Med* 6, 439-444.

Wadhwa, R., Yaguchi, T., Hasan, M. K., Mitsui, Y., Reddel, R. R., and Kaul, S. C. (2002). Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. *Exp Cell Res* 274, 246-253.

Wagner, I., Arlt, H., van Dyck, L., Langer, T., and Neupert, W. (1994). Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *Embo J* 13, 5135-5145.

Werner-Washburne, M., and Craig, E. A. (1989). Expression of members of the *Saccharomyces cerevisiae* hsp70 multigene family. *Genome* 31, 684-689.

Werner-Washburne, M., Stone, D. E., and Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 7, 2568-2577.

Westermann, B., Prip-Buus, C., Neupert, W., and Schwarz, E. (1995). The role of the GrpE homologue, Mge1p, in mediating protein import and protein folding in mitochondria. *Embo J* 14, 3452-3460.

Wiedemann, N., Frazier, A. E., and Pfanner, N. (2004). The protein import machinery of mitochondria. *J Biol Chem* 279, 14473-14476.

Wilks, J. C., and Slonczewski, J. L. (2007). pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. *J Bacteriol* 189, 5601-5607.

- Willmund, F., Hinnenberger, M., Nick, S., Schulz-Raffelt, M., Muhlhaus, T., and Schroda, M. (2008). Assistance for a chaperone: *Chlamydomonas* HEP2 activates plastidic HSP70B for cochaperone binding. *J Biol Chem* 283, 16363-16373.
- Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., *et al.* (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285, 901-906.
- Yamamoto, H., Momose, T., Yatsukawa, Y., Ohshima, C., Ishikawa, D., Sato, T., Tamura, Y., Ohwa, Y., and Endo, T. (2005). Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane. *FEBS Lett* 579, 507-511.
- Yi, X., Luk, J. M., Lee, N. P., Peng, J., Leng, X., Guan, X. Y., Lau, G. K., Beretta, L., and Fan, S. T. (2008). Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. *Mol Cell Proteomics* 7, 315-325.
- Yokoyama, K., Fukumoto, K., Murakami, T., Harada, S., Hosono, R., Wadhwa, R., Mitsui, Y., and Ohkuma, S. (2002). Extended longevity of *Caenorhabditis elegans* by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. *FEBS Lett* 516, 53-57.
- Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nat Rev Mol Cell Biol* 5, 781-791.
- Young, J. C., Barral, J. M., and Ulrich Hartl, F. (2003). More than folding: localized functions of cytosolic chaperones. *Trends Biochem Sci* 28, 541-547.
- Zhang, Y., and Zuiderweg, E. R. (2004). The 70-kDa heat shock protein chaperone nucleotide-binding domain in solution unveiled as a molecular machine that can reorient its functional subdomains. *Proc Natl Acad Sci U S A* 101, 10272-10277.
- Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* 272, 1606-1614.